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Stereochemical studies in the α-aryl[1] carboxylic acid series with special reference to phenyl- and 2-thienyl-substituted compounds

By KURT PETTERSSON

With 10 figures in the text

Chemical reactions between asymmetric compounds occur frequently in the field of biochemistry. A study of these reactions should therefore give valuable information about biological processes and an increasing understanding of such important problems as metabolism and drug action. But even from a purely chemical viewpoint these reactions are of considerable interest, not only because they afford an increasing knowledge of this interesting class of compounds, but also because they constitute valuable aids for the study of more general problems as, for example, reaction mechanisms.

An important question concerning asymmetric compounds is their configurational relation which may be determined by chemical and physical methods. Although the problem of absolute configuration has been solved in principle by Bijvoet (11), it is generally more convenient to determine the configuration of a compound relative to that of a given standard substance, usually D-glyceraldehyde. Since the absolute configuration of this substance is known from the work of Bijvoet, it follows that the absolute configuration of compounds related to this standard is also known.

Correlation of compounds containing a phenyl group directly linked to an asymmetric carbon atom and aliphatic compounds often presents some difficulties (12, 13, 14), and at the beginning of the present work only a few α-phenyl carboxylic acids had been related to D-glyceraldehyde (15). A method for establishing the mutual relation within the group has been proposed by Kuhn (16). The method is based upon the assumption that the phenyl group has a dominating influence on the optical activity of the compounds, and therefore those compounds which have the same mode of rotation have also the same configuration.

Analogously constituted phenyl and thienyl compounds often show far-reaching similarities in their physical and chemical behaviour. It seemed to be of interest, therefore, to compare the properties of analogously constituted compounds containing phenyl and 2-thienyl groups directly attached to an asymmetric center. Since phenyl and thienyl compounds are often isomorphic, it seemed highly probable that the steric relations could be determined by means of the quasi-racemate method. A comparison of the influence of the aromatic groups on the optical activity of the compounds and their ability to form racemates should also throw some light on these interesting phenomena.

[1] Carbocyclic as well as heterocyclic groups with aromatic properties are here considered as aryl groups.

Material and methods of preparation

The compounds studied may be classified in three different groups: The α,β-diarylpropionic acids (arylmethyl-arylacetic acids), the arylsuccinic acids and the α-phenyl fatty acids (alkylphenylacetic acids). The formulae of the compounds are collected in Fig. 1.

The members of the first group consist of the four acids obtained if the two aryl groups possess the four possible combinations of phenyl and 2-thienyl (I, II, III, IV) (17). In general, these acids may be obtained by alkylation of the corresponding arylacetonitriles (18) or ethyl arylmalonates (19, 20), followed by hydrolysis to the acids and decarboxylation of the malonic acids. However, it is often more convenient to alkylate the esters of the arylcyanoacetic acids, since these reactions often give higher yields and more pure products. For that purpose ethyl 2-thienylcyanoacetate was prepared by carbethoxylation of 2-thienylacetonitril (1, 94), and the alkylation of the ester as a method for preparation of α-(2-thienyl)-substituted acids was demonstrated (1, 2, 17).

The optical resolution of the diarylpropionic acids were successful except in the case of the dithienylpropionic acid (III) (of which only one of the antipodes could be obtained) (4, 5). There are usually three criteria of optical purity: The constancy of the optical activity upon further recrystallisations of the alkaloid salt, a sharp melting point of the compound and agreement between the optical activities of the antipodes obtained with different alkaloids. If, however, the antipodes form a continuous series of mixed crystals, as in the case of the dithienylpropionic acid, a small melting-point interval is no indication of optical purity. Since the rotation of the (−)-acid was considerably higher than that of the (+)-acid, the third criterion fails also. It is probable, however, that the (−)-α,β-di(2-thienyl)propionic acid obtained is at least not very far from optical purity (*vide infra*).

2-Thienylsuccinic acid (XII) was prepared mainly according to three general methods: (i) Carbethoxymethylation of ethyl 2-thienylcyanoacetate, followed by decarboxylation and hydrolysis of the resulting ethyl α-(2-thienyl)-α-cyanosuccinate (2); (ii) addition of potassium cyanide to ethyl 2-thenalmalonate, alkaline hydrolysis of the resulting ethyl β-(2-thienyl)-β-cyanopropionate to the corresponding acid and subsequent hydrolysis of the cyanoacid in boiling water (3); (iii) addition of potassium cyanide to ethyl α-cyano-β-(2-thienyl)acrylate, followed by acid hydrolysis of the resulting ethyl α,β-dicyano-β-(2-thienyl)propionate in a mixture of ether and hydrochloric acid (3).

The physical properties of the optically active 2-thienylsuccinic acids indicate that the resolution is complete. The antipodes as well as the optically inactive acid decompose at the melting points, probably forming anhydrids. In addition, the antipodes racemize under various conditions (3).

The five lowest members of the α-phenyl fatty acid series from hydratropic acid to α-phenyloenanthic acid were resolved into optical antipodes and used as intermediates in the correlation of the diarylpropionic acids to D-glyceraldehyde (Fig. 1, formulae V through IX) (8, 10). As far as the author knows, the three higher members of the series have not been fully resolved earlier. The resolutions were performed with the enantiomorphs of α-phenylethylamine. It is interesting to note that hydratropic acid differs from the four other acids in so far as the (+)-acid is obtained with the *dextro*-rotatory amine, whereas of the other acids the (−)-acid is obtained. The optically active acids, except those of hydratropic acid, could not be crystallized.

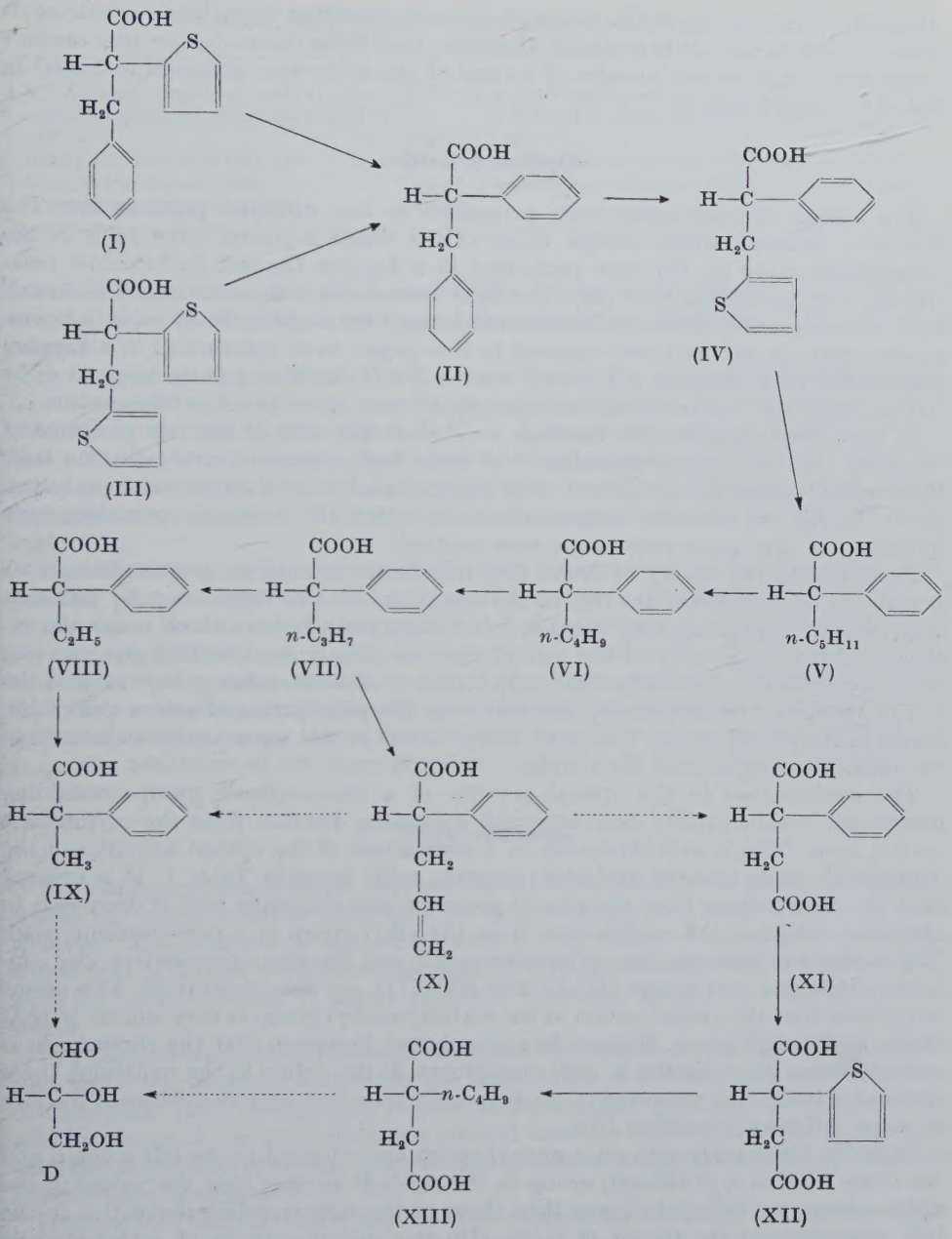


Fig. 1. Sterically related acids. All of the compounds are *dextro*-rotatory. Relations not determined by the author are indicated by dotted arrows.

Attempts to initiate crystallisation with the corresponding crystalline racemic acids were without success. It is probable, therefore, that these racemates are true racemic compounds. Amides and anilides of several of the acids were prepared and used in the steric investigations.

Optical activities

The optical measurements were performed in two different polarimeters. The definitive determinations except those of the liquid α-phenyl fatty acids in the homogenous state (8, 10) were performed in a Lippich-Landolt half-shadow polarimeter with petroleum bath (21). The light source was a conventional 6 V electric bulb with bayonet-type base. The measurements of the α-phenyl fatty acids in homogenous state, as well as those reported in this paper, were determined in a Lippich polarimeter from Schmidt & Haensch (model 14174) with a sodium lamp as light source. With this apparatus the measurements were made at room temperature.

In each determination five readings were made for each of the two positions of the tube. The zero point correction was made from measurements with the tube filled with the appropriate solvent. The solvents used were of commercial analytical grade. In the less accurate measurements, by which the resolution processes were followed, the zero point corrections were omitted.

According to the theory of Kuhn (22) the groups around an asymmetric center contribute to the optical activity of the compound in two fundamentally different ways. Firstly, the group may contain active absorption bands, which under the influence of the asymmetry of the rest of the molecule (vicinal action) give rise to a direct contribution. Secondly, the group together with two other groups around the center provides the asymmetry necessary for the polarization of active absorption bands in the fourth group. The most active bands in this sense are those located in the ultraviolet region near the visible.

The contribution to the optical activity of a chromophoric group containing anisotropic bands, rapidly decreases with increasing distance from the asymmetric carbon atom. This is well illustrated by a comparison of the optical activities of the arylsuccinic acids and the arylmethylsuccinic acids given in Table 1. It is evident that the contribution from the phenyl group of benzylsuccinic acid is decreased to the same extent as the contribution from the alkyl group in *n*-pentylsuccinic acid. The similarities between the arylsuccinic acids and the diarylpropionic acids, containing the same aryl groups (II, XI and III, XII), are also remarkable. This seems to indicate that the vicinal action of the carboxymethyl group is very similar to that of the arylmethyl group. It must be remembered, however, that the comparison is made between the activities in only one solvent. If the values in the remaining three common solvents are compared (5, 6), it is easily seen that only the values of the ions in water differ by more than 10 %.

In Table 1 the acids with an α-phenyl group are collected in the left column and the acids with an α-(2-thienyl) group in the right. It is clear that the values in the right column are definitely lower than those of the corresponding derivative in the left. According to the theory of Kuhn (16) the optical activity of α-phenylacetic acids in the visible region is predominantly determined by the phenyl band located at about 2500 A. It is tempting to assume that the 2-thienylacetic acids behave in a similar manner and that the optical activity of these compounds is mainly determined by the thienyl absorption band, probably located at about 2300 A (26). Whether the

Table 1. $[M]_D^{25}$ of α-aryl carboxylic acids in ethanol.

α,β-diphenylpropionic acid (II) (5)	277°	α,β-dithienylpropionic acid (III) (5)	196°
α-phenyl-β-thienylpropionic acid (IV) (4)	265°	α-thienyl-β-phenylpropionic acid (I) (4)	181°
phenylsuccinic acid (XI) (6)	280°	2-thienylsuccinic acid (XII) (6)	206°
benzylsuccinic acid (23)	43.5°	2-thenylsuccinic acid (24)	16.5°
n-pentylsuccinic acid (25)	49.4°		

lower activities of the thiophene compounds depend on the shorter wave-length of absorption or on smaller anisotropy of the absorption band cannot be settled at present.

In the preceding paragraph it was assumed that the (−)-α,β-dithienylpropionic acid obtained is at least not very far from optical purity. An indication of this is afforded by the optical activities in Table 1. A comparison of the values of the three first acids in the two columns shows that dithienylpropionic acid occupies about the same place between α-thienyl-β-phenylpropionic acid and thienylsuccinic acid as diphenylpropionic acid between α-phenyl-β-thienylpropionic acid and phenylsuccinic acid.

Since the thienyl compounds evidently have lower optical rotation than the corresponding phenyl compounds one could perhaps expect that dithienylpropionic acid should have lower rotation than α-thienyl-β-phenylpropionic acid. However, the β-aryl groups probably act mainly through their vicinal action on the α-aryl group, and the vicinal action is caused by a state of coupled electrons. It is quite probable that such a coupling is more readily obtained between two similar aromatic groups with their similar energy states than between two different aromatic groups. If this is so, the behaviour of the diarylpropionic acids should be as shown in Table 1.

Steric relations

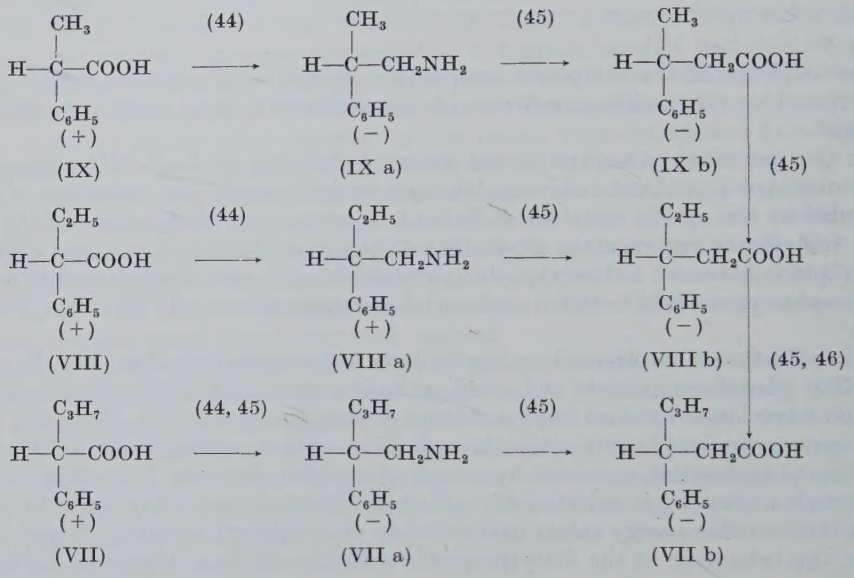
The configurational relation between the acids investigated is shown in Figure 1, where the steric formulae according to the convention of Emil Fischer (27) are collected. The sequence of substances ends in two points with compounds (hydratropic acid (IX) and *n*-butylsuccinic acid (XIII)), previously related to glyceraldehyde by other authors. It seems justified, therefore, to shortly summarize these works.

In a thermal study of alkylsuccinic acids and alkylsulfidesuccinic acids Matell found that the *n*-alkylsuccinic acids with the same mode of rotation have also the same configuration (28). Fredga (29) related methylsuccinic acid and mercaptosuccinic acid by means of the quasi-racemate method and in a similar way mercaptosuccinic acid and malic acid (30). The latter acid was related to glyceraldehyde through the intermediate tartaric acid (31, 32).

Hydratropic acid has been converted to α-phenethylamine by several rearrangement reactions, known to proceed with retention of configuration (33, 34, 35). Oxidative degradation of the phenyl group in the amine yields alanine (12), related to lactic acid (36, 37, 38). Mislow related hydratropic acid and lactic acid through mandelic acid (39, 14). Lactic acid is related to glyceraldehyde through isoserine (40, 41). Fredga related hydratropic acid and allylphenylacetic acid (X) (42) which

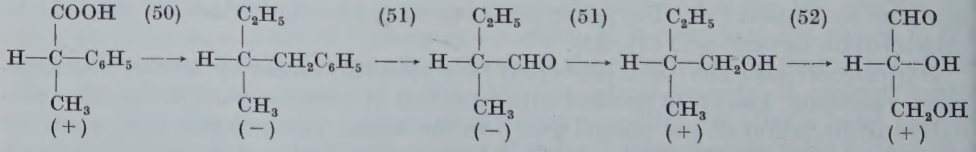
was oxidized to phenylsuccinic acid (43). The last-named acid is related to glyceraldehyde as shown in Figure 1.

Levene *et al.* have published, in a series of papers (44–48), a steric investigation including among other substances hydratropic acid (IX) and its two next homologues (VIII, VII). For the purpose of convenience the main relations are shown in the scheme below (compare 48). Frequently occurring formal errors in the papers as well as repeated



corrections of the signs of rotation (46, 47) leave the reader somewhat uncertain as to the correctness of the results. It seemed to be of interest, therefore, to test the very surprising relation between the amines in the second column (VII a–IX a). The amides of the optically active phenyl fatty acids (VII–IX) were hydrogenated to the corresponding amines by means of lithium aluminium hydride (see experimental part). The sign of rotation of the amines was found to be in accordance to those obtained by Levene *et al.* It seems to the author that a study of the rotatory dispersion of these amines as well as their homologues would be of interest, since alternating rotatory powers in homologous series of this kind are rather unexpected.

Evidence for the D-configuration of the *dextro*-rotatory hydratropic acid may be found in the enormous steric work by Levene *et al.*, if their "rules of masses" are correct (which is evidently not the case for the amines discussed above) (compare 49):



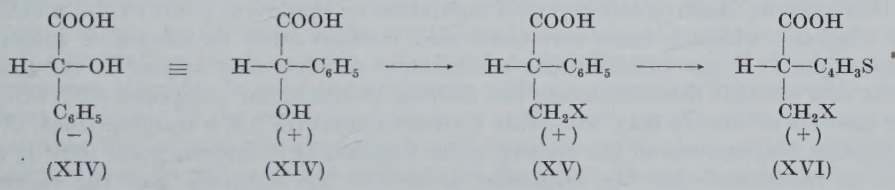
The steric relation between the diarylpropionic acids (I), (II) and (IV) was established by means of the quasi-racemate method and by comparison of the optical

activities in different solvents (4, 5, 7. *Vide infra*). The conclusion about the configuration of α,β-di(2-thienyl)propionic acid (III) is based only on the results of the optical method (5).

Reductive desulfurization of the thienyl group in optically active α-phenyl-β-(2-thienyl)propionic acid (IV) yields active α-phenyloenanthic acid (V) (9) and correlation between the configurations of the compounds of the diarylpropionic acid series and those of the α-phenyl fatty acid series was thus obtained. The members of the last-mentioned series were connected amongst themselves by the quasi-racemate method applied to the amides and by the optical displacement rule (9, 10. *Vide infra*). Allylphenylacetic acid (X) was connected to α-phenyl-*n*-valeric acid (VII) by catalytic hydrogenation of the allyl double bond (9).

The arylsuccinic acids (XI, XII) were correlated by means of the quasi-racemate formed between the acids with opposite mode of rotation (6). Reductive desulfurization of the aromatic group in optically active 2-thienylsuccinic acid yielded *n*-butylsuccinic acid (XIII) with the same direction of rotation (3).

All acids except (XIII) were related by comparison of their optical activities in different solvents. It is interesting to note that the method, proposed by Kuhn (16) and briefly mentioned in the introduction, is applicable not only to the substituted phenylacetic acids investigated but also to the analogous 2-thienylacetic acids. (The succinic acids may, of course, be considered as substituted acetic acids). It should be pointed out that the relations, shown in the formulae XIV, XV and XVI, give a possible but less common correlation between mandelic acid and glyceraldehyde.



Stereochemical methods

Chemical methods

As is well known, a reaction which does not involve the rupture of a bond to an asymmetric carbon atom in general proceeds with retention of the configuration. This is always the case if only one of the groups around the center is attacked. The reactions referred to in this paper are of this type.

As was pointed out in the introduction a correlation of aromatic and aliphatic compounds by chemical means is often difficult to perform (12, 13, 14). Physical methods such as the quasi-racemate method or optical methods are probably not applicable because of the great structural differences between such compounds. Therefore, the convenient method described by Fredga in 1953 was very opportune (25). According to this method the thiophene nucleus is desulfurized with Raney nickel, forming the corresponding aliphatic compound. Since phenyl and thienyl compounds often give quasi-racemates (6, 7, 24, 53) the method is very useful for the correlation of phenyl compounds to glyceraldehyde. In this way α-phenyl-β-(2-thienyl)propionic acid (IV) was converted to α-phenyloenanthic acid (V) (9) and 2-thienylsuccinic acid (XII) to *n*-butylsuccinic acid (XIII) (3).

It is perhaps somewhat superfluous to mention that the desulfurization process is by no means limited to thiophene compounds. However, a more special application of the method may be emphasized. As will be discussed more thoroughly in the following paragraph the possibilities for two compounds to form quasi-racemates depend on the structural similarity between them. Fredga has shown that the differences may be reduced, if a common large substituent is introduced into both substances (36, 54, 55, 56). Sulfur-containing groups are often easily introduced (as for example a phenylmercapto group) and also easily removed by means of Raney nickel.

Catalytic reduction of the allyl double bond in allylphenylacetic acid (X) yielded the corresponding α-phenylvaleric acid (VII) (9).

The quasi-racemate method

1. RACEMATE FORMATION.—The optically inactive substance, obtained from equimolar quantities of two optical antipodes may, if solid, be classified in one of three different groups, depending on its crystal behaviour (57). Most commonly a molecular compound, i.e. a racemate, is formed, which has physical properties different from those of the enantiomers and may be detected by different physical methods. A melting-point diagram of such a system shows, in general, the molecular compound as a maximum, surrounded by two symmetrically situated eutectic points. More rarely, the inactive substance is a solid solution of the antipodes (a pseudo-racemate), and crystallizes in the same crystal form as these. The melting-point diagram shows in this case the inactive solution as a maximum or minimum point in the middle of the diagram, which in some rare cases may contain other maximum or minimum points (58). It is also possible that the inactive solution may appear as the middle point of a straight line connecting the melting points of the antipodes (59). Finally, the inactive substance may constitute a eutectic mixture, i.e. a conglomerate, of the antipodes, and appears in the melting-point diagram as a eutectic point (60). It may also be mentioned that the antipodes as well as the antipode plus the racemate (10, Figs. 1 and 2) may show more or less far-reaching solid solubility. This fact seems to be but little discussed in the literature and is scarcely noticed in the usual textbooks.

Little is known about the factors which cause the inactive substance to crystallize in a certain form, for example as a racemic compound. Probably these factors are also responsible for the stability of the racemates, and this stability in turn seems to be of great importance for the question of quasi-racemate formation (*vide infra*). Landrieu (61) advanced the view that compounds such as acids and salts which are able to form ions should also tend to form true racemates, whereas undissociable compounds should form pseudo-racemates. Ross and Somerville (62) found that strongly associating substances give racemic compounds and feebly or not associating substances give pseudo-racemates or conglomerates. On the other hand Matell (60) found that β-oxy-β-phenylpivalic acid gives a conglomerate, whereas its methyl ester gives a true racemate.

The stability of a racemic compound is indicated by the energy change in the reaction

$$l\text{-A} + d\text{-A} = rac.\text{-A}$$

which may be determined calorimetrically. However, more convenient methods are desired and, therefore, the height of the maximum in the melting-point curve, the

extent of the middle branch of the curve or the difference between the melting point of the racemate and the transition point, beyond which the racemate is unstable, have frequently been used as indications of the stability of the racemate. Findlay and Campbell (63) found that the stability of racemic acid (optically inactive dihydroxysuccinic acid) is about the same as that of its methyl ester, whereas acylation of the hydroxyl group of the ester considerably decreased the stability of the racemate. On the other hand, acylation and esterification of mandelic acid caused respectively a slight and a considerable increase in this stability (64).

Since the results summarized above are in part contradictory and inconsistent it seems to the author to be of value to note every relation between structure and racemate-formation tendency that might be found. A collection of experimental facts could perhaps complete the picture of this particular type of molecular compound, the formation of which is still little understood. In fact, the present material seems to be well suited for this purpose, since it consists in part of a homologous series (9, 10), making it possible to follow the influence of a continuous change in one of the groups around the asymmetric center, and in part of compounds in which a phenyl group has been replaced by a 2-thienyl group (4, 5, 6). Since these groups are known to have similar properties in many respects, it is of interest to find out if an interchange of the groups in different compounds is reflected in the racemate-formation tendency.

The homologous series consists of the amides of the five lowest members of the α-phenyl fatty acid series (V–IX). Unfortunately, the behaviour of the corresponding acids is not known with certainty, since, as mentioned above, only the antipodes of hydratropic acid are obtained in the crystalline state. It is rather probable, however, that the crystalline inactive acids (all except α-phenylcaproic acid) are true racemates, since attempts to seed the antipodes with the corresponding racemate were without success[1]. If the crystalline inactive acids were pseudo-racemates or conglomerates, they should contain crystals with the same crystal form as the antipodes, and should then be well suited as seedlings for the latter. Therefore, it seems justified to suppose that the inactive acids are true racemates with, as it seems, considerably higher melting points than the antipodes. If this is so, the stability of the racemates is lowered by conversion to the amides (9, 10).

As already mentioned, the racemate-formation tendency may be indicated by the distance between the eutectics or the difference in melting point between the racemate and the eutectic, and also by the difference between the racemates and the antipodes (63). Usually these measurements give indications in the same direction, for example, a great racemate-formation tendency corresponds to a large distance between the eutectics, a large difference between the melting points of the racemate and the eutectics, and a large positive difference between the melting points of the racemates and the antipodes. A small racemate-formation tendency corresponds to a small distance between the eutectics, a small difference between the melting points of the racemate and the eutectics and so on. In the case of hydratropamide the relations are somewhat uncertain (10). The inactive amide is a true racemate, melting only about 1.5° higher than the eutectics, indicating a low ability of racemate formation. The middle branch of the curve occupies about half of the diagram, indicating a moderate tendency for racemate formation and the difference of −7° between the racemate and the antipodes indicate a comparatively low tendency. It seems to the

[1] The results of Pickard and Yates (91) concerning α-phenylvaleric acid could not be reproduced (8).

author that if a comparison between several compounds is performed, it should be better to consider the height of the maximum *in comparison to* the difference between the melting points of the antipodes and the eutectics. A ratio less than 0.5 could then be classified as a small racemate-formation tendency, a ratio between 0.5 and 1.5 as moderate and a ratio greater than 1.5 as strong. These values usually correspond to a distance between the eutectics of less than 50 %, 50–75 % and more than 75 % of the diagram, respectively. Of course, this classification is quite arbitrary, but it might give a substantiation of the expressions small, moderate and great racemate-formation tendency used in the text.

The stability of the racemates of the homologous alkylphenylacetamides (9, 10) varies in an irregular manner. The lowest stability is shown by the racemic α-phenylvaleramide, which is a little less stable than hydratropamide. The racemate-formation tendency of these compounds must be classified as small. More stable are the racemic α-phenylbutyramide with a moderate and the racemic α-phenylcaproic and α-phenyloenanthic amides with a great tendency for racemate formation. It is obvious that substances belonging to a homologous series may show considerable differences in the racemate-formation tendency (compare also 56). Since the association tendency of the amides in question is of at least the same order of magnitude and probably varies in a regular manner, the results indicate that factors other than the association ability are also involved in the racemate formation.

If the diarylpropionic acids (I, II, III, IV) are considered (4, 5), one finds that the racemate-formation tendency decreases in the order α,β-diphenylpropionic acid (great) > α-thienyl-β-phenylpropionic acid (moderate) > α-phenyl-β-thienylpropionic acid (small) > α,β-dithienylpropionic acid, which does not form a true racemate at all. The exchange of a phenyl group for a 2-thienyl group evidently affects the ability for racemate formation to an extent which *cannot be explained by the difference in the association tendency*. Furthermore, the association of the dithienyl compound should be greater than for the diphenyl compound because of the expected greater acidity, and the results are then contradictory to the theory of Ross and Somerville (62).

If two optically active acids are isomorphic, one could perhaps expect that they should also have similar capacities to combine with their antipodes to form racemic compounds. It is well known that analogously constituted compounds, containing phenyl and thienyl groups, are often isomorphic. If the tendency of forming racemates is only or predominantly a crystallographic property, one could then expect that the racemate-formation tendency should be of the same order for the thienyl and phenyl compounds. Among the substances under discussion the arylsuccinic acids are isomorphic (6), but unfortunately this case is complicated by polymorphism. It is remarkable, however, that the more stable modification of the optically inactive phenylsuccinic acid is a true racemate, whereas the single known modification of the inactive 2-thienylsuccinic acid is very likely a pseudo-racemate.

An inspection of the behaviour of the diarylpropionic acids from this point of view affords some interesting observations. The acids may be paired in three different groups in respect to the groups around the asymmetric center: (1) those which have the same aryl group in the α-position but different groups in the β-position; (2) those which have different aryl groups in the α-position but the same group in the β-position; and (3) those with different aryl groups in both the α- and β-positions. In the first group we have the two pairs α,β-diphenylpropionic acid (II)–α-phenyl-β-thienylpropionic acid (IV) and α-thienyl-β-phenylpropionic acid (I)–α,β-dithienylpropionic

acid (III); in the second group the two pairs α,β-diphenylpropionic acid (II)–α-thienyl-β-phenylpropionic acid (I) and α-phenyl-β-thienylpropionic acid (IV)–α,β-dithienylpropionic acid (III); and in the third group the pairs α,β-diphenylpropionic acid (II)–α,β-dithienylpropionic acid (III) and α-phenyl-β-thienylpropionic acid (IV)–α-thienyl-β-phenylpropionic acid (I). Since an aryl group in the α-position has fewer degrees of freedom in respect to the asymmetric center than in the β-position, one should expect that the paired substances in the first group should show more similar properties than those of the other groups. This is indeed the case. The first pair of the first group is isomorphic (7, Fig. 3), whereas the second pair with some probability is isodimorphic (7, Fig. 5). In the other groups some pairs show far-reaching solid solubility, but in no case is there any indication of crystallographic similarity (4, 5, 7). If, now, the racemate-formation tendency is in some way related to the crystallographic behaviour, the paired substances of the first group should resemble each other in their ability to form racemates, i.e. α-phenyl-β-thienylpropionic acid (IV) should have greater ability for racemate formation than α-thienyl-β-phenylpropionic acid (I). But from Figs. 1 and 2 in (4) it is evident that the facts are quite the contrary and again no relation between isomorphism and racemate-formation tendency could be found. The differences between α,β-dithienylpropionic acid and α-thienyl-β-phenylpropionic acid are noteworthy since their antipodes are probably isodimorphic, whereas the optically inactive forms are so clearly different, the first being a pseudo-racemate and the second a true racemate with moderate stability.

Hitherto the only compounds considered are those with isomorphic enantiomers. Still more reliable results would be obtained if the racemic compounds were also isomorphic. This is not the case for the arylsuccinic acids which could be investigated with X-rays (6). Neither has a complete investigation of isomorphism between the racemic diarylpropionic acids been performed, but a thermal investigation of contact preparations showed that only α,β-diphenylpropionic acid (II) and α-thienyl-β-phenylpropionic acid (I) could possibly be isomorphic. However, benzylsuccinic acid and the two isomeric thenylsuccinic acids fulfil these requirements (24, 53). The tendency for racemate formation decreases in the order 2-thenylsuccinic acid (great) > benzylsuccinic acid (moderate) > 3-thenylsuccinic acid (small). Thus, the order between the phenyl and 2-thienyl compounds is reversed in comparison to the compounds discussed above, but even here the exchange of the phenyl group for a thienyl group appreciably affects the stability of the racemates.

From what has been said it is evident that the formation of racemates is a complicated phenomenon, at least not predominantly depending on either the association tendency or the crystallographic properties. It is assumed that it must be possible to bring the molecules of the two antipodes in a true racemic compound into coincidence by means of a symmetry operation (65). It has also been pointed out that the condition for two antipodes to form solid solutions is that the structure of the antipodes must not differ too much from a structure which is identical with its mirror image (65). Both these conditions depend on the structural framework of the molecule. Therefore, the differences between the phenyl- and 2-thienyl-substituted compounds are interesting, since these groups are similar chemically and have similar space requirements. It is possible that the forces arising from the sulfur atom may have an influence which may be difficult to predict. In the substituted succinic acid series a cyclized substituent seems to have a dominating influence, since the racemate-formation tendency decreases from excellent for cyclohexylsuccinic acid (58) to zero for 2-thienylsuccinic acid. On the other hand the tendency of the alkylsuccinic

acids from methyl to hexyl is of the same order, the racemates of the acids with an even number of carbon atoms in the alkyl chain being a little more stable than the others (28). It is possible, that the length of the alkyl groups is also responsible for the irregular variation of the stability of the alkylphenylacetamides. In fact, a minimum is obviously present for the *n*-propyl derivative in the alkylsuccinic acid series also (28).

2. QUASI-RACEMATE FORMATION.—The only difference between the components of a racemate is the configuration. One might ask what happens, if structural differences are also introduced. Already in 1899 Centnerszwer made the interesting observation that equimolar quantities of (+)-chlorosuccinic acid and (−)-bromosuccinic acid give a molecular compound (66). Since these acids are known to have opposite configurations (compare 56) the molecular compound must be considered as a kind of racemic compound, a quasi-racemate (67). The existence of such compounds was discussed by Delépine (68), but although this author clearly pointed out that the components have opposite configurations, the formation of these compounds as an indication of steric relation was first introduced by Timmermans (69), and in the hands of this author (70) and, above all, of Fredga *et al.* (56, 71) the method has been developed to a valuable aid in steric investigations. It is remarkable that this method, which cannot be theoretically derived, as yet has not in any case given contradictory results but has often been applicable where other methods have failed.

A quasi-racemic compound is to be considered as a true racemic compound, in which one of the components is replaced by a similarly constituted compound with the same configuration. It is evident, then, that the factors which determine the formation of the racemate also determine the formation of the quasi-racemate, but in addition, the differences between the replacing compounds must be accounted for. The first-mentioned factors may be summarized as the tendency for racemate formation. The importance of this tendency for the formation of quasi-racemates has been pointed out by Fredga (56), who found that a compound with sufficient ability for racemate formation was able to form molecular compounds with quasi-racemic character even with structurally related symmetric compounds (67). The requirements that must be demanded of a compound replacing one of the components in a racemate were discussed by Lettré (72), who proposed that the interchanging compounds should be isomorphic. However, Fredga (56) emphasized that this was too rigidly formulated, since quasi-racemates were also obtained with compounds which were not isomorphic. Nevertheless, it is a self-evident condition that the structural differences must be limited, if the basic principle for the quasi-racemate is maintained, i.e. the analogy to the true racemate (*vide infra*).

In a stereochemical study on plant growth substances Matell (73) found that the formation of quasi-racemates seems to be "more dependent on structural differences than on the racemate-formation tendency". This conclusion was drawn from a consideration of the occurrence of quasi-racemates as a function of the racemate-formation tendency. Unfortunately, the fact that the formation of a quasi-racemate is dependent on two racemate-formation tendencies was not accounted for. This difficulty is minimized, if the substances to be compared are combined with a properly chosen third substance. An example may illustrate the idea.

α,β-Diphenylpropionic acid (II), α-(2-thienyl)-β-phenylpropionic acid (I) and α,β-di(2-thienyl)propionic acid (III) have great, moderate and no racemate-formation tendency, respectively (4, 5). The structural differences between the two first-men-

tioned acids are probably greater than between the two last-mentioned, since in the first case the antipodes with the same configuration form a diagram with far-reaching solid solubility, whereas in the second case the antipodes are probably isodimorphic (7, Figs. 1 and 5). Now, the first-mentioned acids form a quasi-racemate but the last-mentioned do not, in spite of their smaller structural differences (7, Figs. 2 and 6). In this case then, the quasi-racemate formation is probably a result of the great racemate-formation tendency of α,β-diphenylpropionic acid. However, it seems rather strange to the author that a simple *general* relation should exist between such complicated and indefinite conceptions as the ability of two optical antipodes to combine to a racemate and the structural differences between one of these antipodes and a second compound.

As was pointed out above, it is necessary that a quasi-racemate should have structural similarity to a racemate. This is often manifested in that it is isomorphic to one or both of the racemates formed of its components (53, 54, 68, 74, 75). If these racemates are not isomorphic, it seems quite possible that the enantiomers with opposite configuration may adjust themselves to a molecular compound with a crystal lattice something between those of the racemates but has, nevertheless, the properties of a quasi-racemate. In such cases it may be difficult to find evidence that the compound is in fact a quasi-racemate. However, it is usually enough to show that an optically active compound gives a molecular compound with equimolar quantities of one enantiomer of another compound but not with the other. It seems to the author desirable that in such cases the structural differences between the compounds should not be too great for the following reason:

A quasi-racemate may be considered as one of two diastereomers, and the mode of detection described above is applicable only in those cases where the second diastereomer does not exist under the experimental conditions used. If the structural differences between the components are great, as for example between acids and bases, the physical properties of the diastereomers may bear no relation to the configuration of the components. This is illustrated by the failure of the rule of Winther (76). According to this rule the antipodes of different acids have the same configuration, if they are precipitated with the same alkaloid. Many cases are reported, however, where this rule fails (77, 78) and another example of this is afforded by the α-phenyl fatty acids (8, 10). It is then quite possible that the enantiomers with the same configuration form a molecular compound, but the enantiomers with opposite configuration do not (compare 54). Therefore, the fact that a molecular compound is formed with one pair of the antipodes but not with the second is not a sufficient condition for this compound to be a quasi-racemate. If, however, the structural differences between the compounds are not too great, the probability that the compound is not the quasi-racemate is rather small, but it seems to the author that in doubtful cases one should try to find crystallographic relations between the molecular compound and the racemates (compare 65).

The conception of the quasi-racemate as one of the two possible diastereomers may also explain the fact that in at least two known cases, the antipode of one compound forms molecular compounds with both antipodes of another (10, 79). The second compound is then the second diastereomer, and it remains to find a relation between one of the compounds and one or both of the racemates. The conditions may be illustrated with an example.

In a previous paper it was shown that (−)-α-phenylbutyramide gives molecular compounds with both antipodes of hydratropamide (10, Figs. 3 and 4), but no attempts

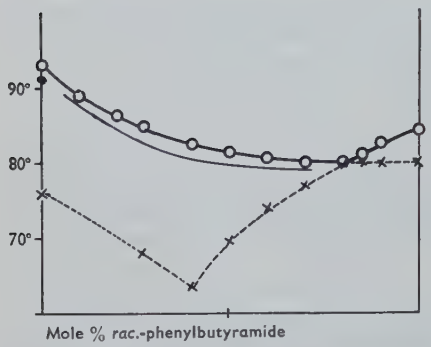


Fig. 2. Melting-point diagram for the system racemic α-phenylbutyramide and the molecular compound, consisting of equimolar parts of (+)-hydratropamide and (−)-α-phenylbutyramide.

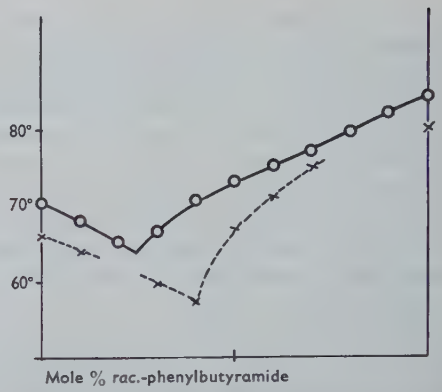


Fig. 3. Melting-point diagram for the system racemic α-phenylbutyramide and the molecular compound, consisting of equimolar parts of (+)-hydratropamide and (+)-α-phenylbutyramide.

were made to relate the compounds to the racemates. It was only assumed that the more pronounced maximum for the amides with opposite direction of rotation was an indication of the quasi-racemate. The melting-point diagram of this compound and racemic α-phenylbutyramide is given in Figure 2 of the present paper. It is evident that the compound is isomorphic to a low-melting modification of the phenylbutyramide. That this modification had not been found earlier, is explainable by the fact that it is rather unstable. The details will be discussed more thoroughly in connection with the question of polymorphism. In Figure 3 is given the diagram obtained with the molecular compound of the amides with the same mode of rotation and racemic phenylbutyramide. In this case no indication of crystallographic similarity is present. The complete melting-point diagrams of hydratropamide and the molecular compounds were not investigated, since contact preparations clearly showed no relations in these cases. The earlier assumption, that the molecular compound obtained from the amides of opposite direction of rotation is the quasi-racemate, was therefore evidently correct (10). Applied in this way the quasi-racemate method gives the same steric relation between hydratropic acid and α-phenylbutyric acid as the methods used before.

The quasi-racemate is like the racemate a molecular compound with physical properties different from those of the components. It may then be detected by different physical methods. In the investigation under discussion mainly three methods have been used: thermal analysis, X-ray diffraction, and infrared spectroscopy.

3. Thermal analysis.—The main method for establishing the presence of a quasi-racemate has been the study of melting-point diagrams (7, 9, 10). The apparatus used was a Kofler hot-stage microscope (80) with the tube protecting the thermometer removed (81). The thermometer was calibrated against azobenzene, benzil and acetanilide with the melting points 68°, 95° and 115°, respectively. The apparatus was fitted with polarizer and analyzer except when the diagrams in (7) were determined. The rate of heating was 1–2°/min.

The melting-point samples were prepared by weighing out appropriate amounts

on a torsion balance (maximal weight 10 mg, accuracy 0.02 mg) (4, 5, 7, 9) or by mixing appropriate volumes of stock solutions (10).

The compounds thermally investigated were stable under the conditions used. If not otherwise stated the compounds were considered as thermally stable, if three complete cycles of melting–crystallisation did not affect the melting point.

The melting-point examination of the samples during the melting process affords some advantages (80). The necessary amounts of the sometimes difficultly accessible enantiomeric compounds are appreciably depressed, the thaw-points are more precisely determined and polymorphic modifications are easily detected. Preliminary investigations of the systems by means of contact preparations (80) were made, but since cases are known where molecular compounds failed to crystallize in such preparations (28), the complete diagrams were also investigated.

4. X-RAY DIFFRACTION.—As is well known, simple phase analyses may be performed by means of X-ray powder photographs. The quasi-racemate as a phase different from the components also has a different powder photograph. If the quasi-racemate is isomorphous to one or both of the corresponding racemates (53) the results are easily understood, whereas in other cases the large amount of lines frequently obtained from organic lattices, may make the result difficult to interpret (6).

The X-ray diffraction method is especially of value if the substances in question are thermally unstable (6), but may also be used to verify or complement the results obtained by the thermal method (5, 10). Although polymorphic modifications may obviously be detected with this method, the occurrence of such modifications may also render the results more difficult to explain. An otherwise very unstable lattice may under certain circumstances be stabilized, as is shown in Figure 2, for example. The fact that the quasi-racemate from phenylsuccinic acid and 2-thienylsuccinic acid is not isomorphic to racemic phenylsuccinic acid (6) may possibly be explained in a similar manner.

In the cases where X-ray investigations were performed, the method proved to be successful, since not only the racemates and the quasi-racemates were detected, but also the molecular compound between hydratropamide and α-phenylbutyramide with the same direction of rotation (5, 10). In addition, the pseudo-racemic nature of optically inactive α,β-di(2-thienyl)propionic acid was verified (5).

The X-ray powder photographs were taken in a focusing camera of Guinier type with CuK radiation (45 kV, 25 mA), using an aluminium single crystal with double curvature as monochromator (82). Because of the high intensity and consequently low time of exposure (15–20 minutes) this monochromator is very convenient. A steric relation between two compounds required six exposures (two antipodes, two racemates and two mixtures), and could be readily performed in four hours including the time required for preparation of the two mixtures.

5. INFRARED SPECTROSCOPY.—In a stereochemical study of dicarboxylic acids Rosenberg and Schotte (83) found that in some cases the existence of quasi-racemates may be indicated by their infrared spectra. The convenience of the method justifies its application to known systems, through which it is possible to find out its limitations.

A mixture of equimolar amounts of α,β-diphenylpropionic acid and α-phenyl-β-thienylpropionic acid with the same mode of rotation has an infrared spectrum which is clearly the sum of those of the components. The mixture of the components with opposite mode of rotation has considerably fewer peaks than should be expected

Table 2. Infrared absorption bands of (+)-hydratropamide and (−)-α-phenylbutyramide.

b = broad; vs = very strong; s = strong; m = medium; w = weak; sh = shoulder.

(+)-Hydratropamide			(−)-α-Phenylbutyramide		
Wave-length in μ	Wave-number in cm^{-1}	Character of the band	Wave-length in μ	Wave-number in cm^{-1}	Character of the band
3.03	3300	s	2.98	3360	s
3.17	3150	s	3.18	3140	s
3.35	2990	m	3.38	2960	m
5.13	1950	w	4.30	2325	w
5.31	1885	w	5.12	1955	w
6.02	1660	vs	5.32	1880	w
6.67	1500	s	6.03	1660	vs
6.83	1465	s	6.22	1610	m
6.88	1455	s	6.28	1590	w
7.11	1405	s	6.67	1500	w
7.28	1375	m	6.86	1460	m
7.69	1300	w	7.12	1405	m
7.82	1280	m	7.70	1300	w
7.92	1265	m sh	8.05	1240	w
8.74	1145	m	8.45	1185	w
8.99	1110	m	8.67	1155	w
9.05	1105	m	8.88	1125	w
9.4	1060	m	9.02	1110	w sh
9.72	1030	m	9.05	1105	w
9.96	1004	w	9.34	1070	w
10.06	994.0	m	9.72	1030	w
11.0	909	w	9.99	1001	w
11.7	855	w sh	10.35	966.2	w b
11.85	843.9	m b	11.08	902.5	w
12.2	820	m b	11.31	884.2	w
13.22	756.4	s	11.80	847.5	w
13.50	740.7	s	12.35	809.7	w b
13.75	727.3	m sh	12.97	771.0	w b
14.41	694.0	vs	13.25	754.7	w b
			13.92	718.4	m b
			14.35	696.9	s b

evidently indicating the quasi-racemate shown in Figure 4 of Ref. 7. On the other hand, the enantiomeric α-phenylbutyramides give mixtures with (−)-α-phenyloenanthamide having very similar spectrograms, which are probably the sum of those of the components. However, the thermal and the X-ray methods indicate a quasi-racemate between the amides with opposite mode of rotation (10, Fig. 15). The racemic amides of hydratropic and α-phenylbutyric acids show some interesting features. The racemic hydratropamide appears in the melting-point diagram as a low maximum and shows together with the antipodes far-reaching mutual solubility in the solid phase (10, Fig. 1). This mutual solubility seems to indicate a certain similarity in structure between the racemate and the antipodes (cf. 65), and one should perhaps expect small differences between the infrared spectra. The racemic phenylbutyramide appears in the diagram (10, Fig. 2) as a well-developed maximum and shows little

if any solubility in the antipode, whereas the antipode is a little more soluble in the racemate. Therefore, the differences between the racemate and the antipode seem to be greater in this case than in the former and the spectra should therefore exhibit greater differences. However, the spectra in the first case are very different, whereas the spectra in the second are almost identical.

In Figures 4 through 7 are given the infrared spectra of the optically active hydratropamide and α-phenylbutyramide and of the two molecular compounds derived from these amides (10). The differences between the molecular compound, derived from the amides with opposite rotation, and the components are evident and especially the occurrence of three bands in the N–H stretching region around 3 microns is significant. In the earlier paper dealing with these amides (10), it was suggested that the molecular compound from the amides with the same mode of rotation showed a spectrum which seemed to be the sum of those of the components. A thorough inspection of the spectra reveals, however, that some differences exist in the region between 10 and 14 microns. In particular the absence of the strong peak at 13.5 microns, derived from hydratropamide, seems difficult to explain if the preparation is a mixture of the components.

The results confirm that it is possible to detect quasi-racemates by means of infrared spectral methods which, however, seem to be less general than the thermal and X-ray methods. This may possibly be explained by the fact that the differences measured arise as secondary effects, caused by lattice disturbances of the interatomic forces.

A Perkin Elmer model 21 spectrophotometer, equipped with a sodium chloride prism, was used for the infrared absorption measurements. The samples of the amides were examined as solid pressed potassium bromide plates, according to the method described by Schiedt and Reinwein (84).

6. Polymorphism.—Refined methods of detection have revealed that a large number of organic compounds may exist in several crystalline modifications. This is also true among the compounds dealt with in this paper. It must be pointed out, however, that a search for polymorphic modifications is beyond the scope of this investigation. Therefore, the observations made are merely to be considered as results obtained along with the steric investigations, and must not be regarded as complete. Nevertheless, the study of polymorphic modifications has in some cases given valuable steric information.

As was mentioned earlier, the quasi-racemate between the hydratropamide and the α-phenylbutyramide with opposite mode of rotation is isomorphic to a less stable modification of racemic α-phenylbutyramide (Fig. 2). As this case is important from a theoretical stand-point, it seems justified to discuss it more thoroughly. The lower-melting modification of the racemate is rather unstable, and was obtained only by seeding with the quasi-racemate. A small amount of the latter was melted in under a cover glass at *A* (Fig. 8) and afterwards the racemate was introduced in the usual way (80). After cooling, the crystallized sample showed an unbroken phase between *A* and *D*. On heating, a transition front began to grow from *D* towards *B*. When the temperature was raised to 79° the sample began to melt at *B*, where the transition front was also reached. The melted region broadened to *C*, when the whole region between *C* and *E* melted at 84°. It follows then that the region between *C* and *D* was originally pure low-melting α-phenylbutyramide. The sample was rapidly cooled and after crystallisation the cold preparation was laid on the microscope, preheated

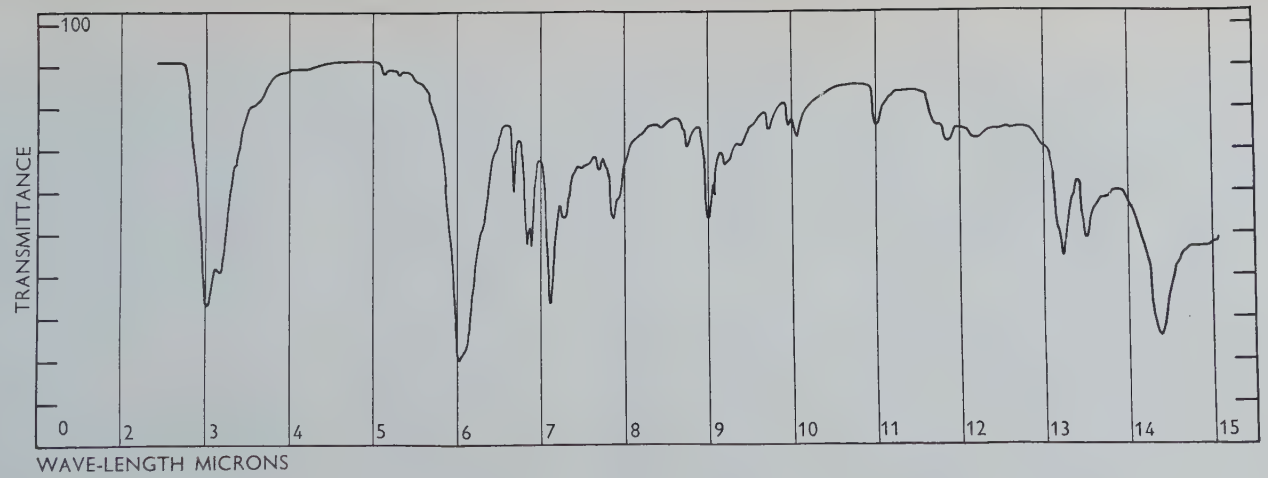


Fig. 4. IR absorption curve of (+)-hydratropamide. KBr phase.

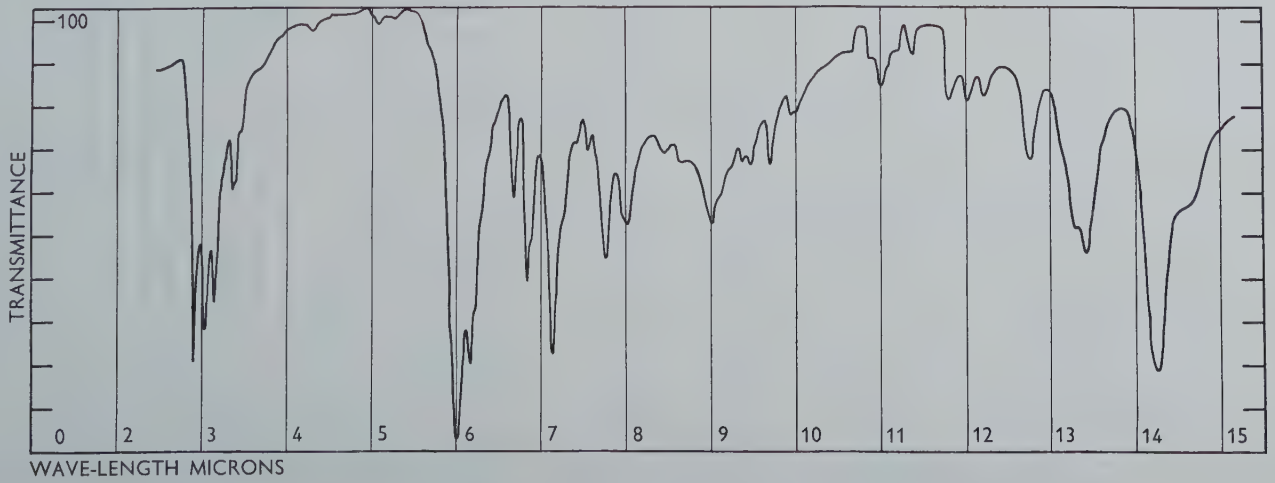


Fig. 5. IR absorption curve of the molecular compound (quasi-racemate), consisting of equimolar parts of (+)-hydratropamide and (−)-α-phenylbutyramide. KBr phase.

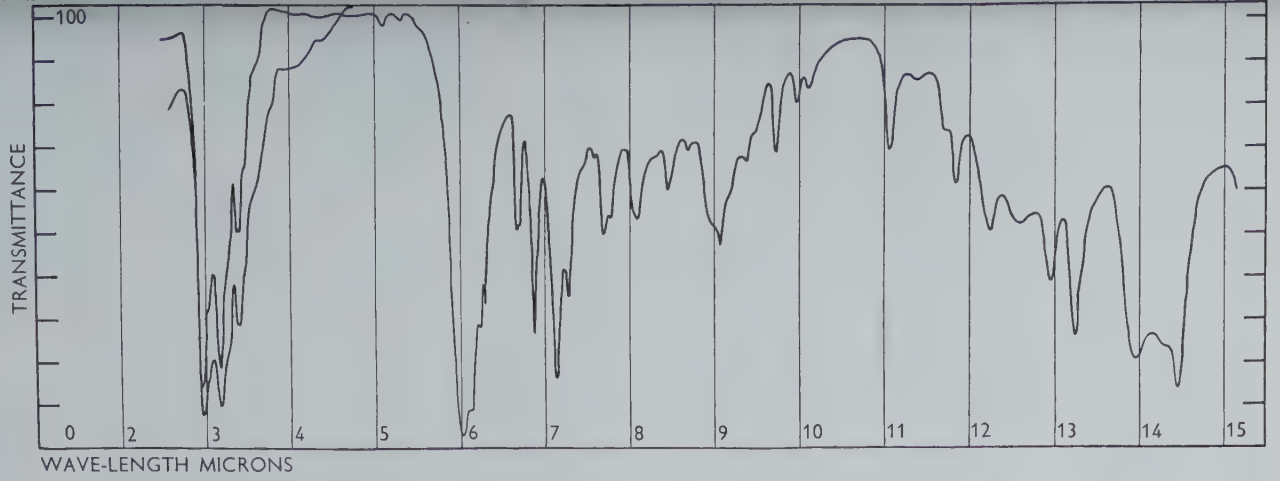


Fig. 6. IR absorption curve of the molecular compound, consisting of equimolar parts of (−)-hydratropamide and (−)-α-phenylbutyramide. KBr phase.

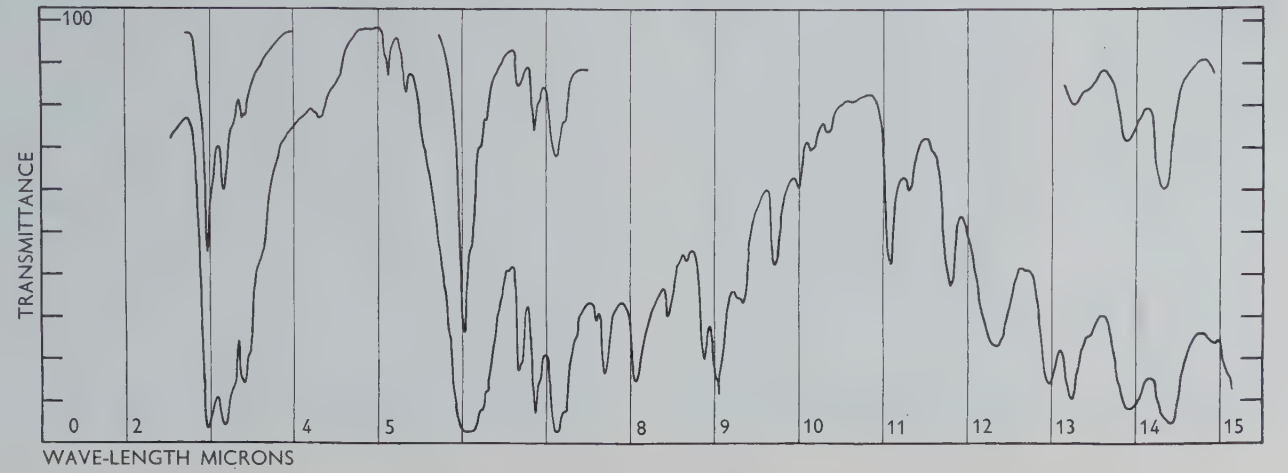


Fig. 7. IR absorption curve of (−)-α-phenylbutyramide. KBr phase.

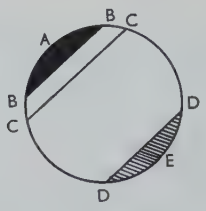


Fig. 8.

to 77–78°. This time the unstable modification between *C* and *D* melted at 80° between rapidly growing crystals of the stable modification.

The lower branch on the left-hand side of the diagram in Figure 2 was obtained only by seeding from point to point from the right-hand side. This may explain why the third modification of the quasi-racemate, constituting the end point of this lower branch, was not obtained earlier (10).

In a previous paper the melting-point diagram of (+)- and (−)-α-phenyl-β-(2-thienyl)propionic acid was reported (4, Fig. 2). A reinvestigation of the racemic acid showed that it could also exist in a second modification, melting at 66.5°. Therefore, the diagram mentioned should be completed with a lower branch between 40 % and 60 % of the (+)-acid, having the maximum at 66.5° and the end points at 65°.

Optical methods

1. The optical displacement rule.—As was emphasized above, the groups around the asymmetric carbon atom contribute to the optical activity of the compound in two different ways: by the anisotropy of their absorption bands and by their vicinal action on the other three groups (22). The vicinal rule of Kuhn (85) says that a substitution in one of the groups mainly affects the anisotropy of the absorption bands of that group, whereas the vicinal action of the group is less affected. The theoretical explanation of the optical displacement rule, formulated by Freudenberg (86), is founded on the vicinal rule. If two similarly constituted compounds with the same configuration undergo similar chemical changes, a shift of the optical activities in the same direction occurs. The reason for this is that the chemical change effects only the contribution from the attacked group and this contribution is about the same in the two compounds. The conditions for the applicability of the displacement rule are determined by the vicinal rule and may be expressed, for example, as follows: (1) the contribution to the optical activity of the groups, which are different in the compounds to be sterically related, must not be very different; (2) the contribution from the attacked groups must be sufficiently great so that a significant shift of the optical activity occurs. The second condition led Freudenberg (87) to the conclusion that a comparison between substituted phenylacetic acids should be very carefully performed, since the optical activity of these compounds are mainly determined by the phenyl group (16).

The α-phenyl fatty acids (V–IX) have been related by the displacement rule applied to the acids, amides and anilides (9, 10). The values are collected in Tables 3 and 4. The optical activities were measured in five different solvents, rendering it less probable that the differences arise as a specific effect of a special solvent. It is evident from the tables that, in fact, the differences between the optical activities of the

acids, amides, and anilides are with a few exceptions significant, and the application of the method is then justified. The reason for this may be that the contributions from the amide and anilide groups are great enough to be clearly reflected in the optical activity of the molecule. Especially the pervading high rotations of the anilides make this assumption reliable. The first-mentioned condition, concerning the differences between the compounds to be compared sterically, is also fulfilled, since *n*-alkyl groups usually have very similar properties. (Compare, however, the amines VIIa, VIIIa and IXa discussed above).

2. Comparison of optical activities in different solvents.—The influence of the solvent on the optical activity of a solute is a very complicated matter (88). Nevertheless, a comparison of the optical activities of two similarly constituted compounds in a series of solvents may give information about the steric relation between them (89, 90). If the optical rotations of the compounds are shifted in the same direction when progressing through the series of solvents, the compounds have the same configuration. If not, no conclusion about the steric relation can be drawn.

The complicated nature of the solvent action makes it obvious that the structural differences between the compounds must be small. It is difficult also to predict how large the differences may be before the method becomes invalid. It seems less surprising, however, that a comparison between, for example, the tetrahydronaphtoic acids and α-phenylvaleric acid fails (91). On the other hand, the reason why a comparison between α-phenoxybutyric acid and α-(1-naphtoxy)butyric acid gives a posi-

Table 3. Optical activities ($[M]_D^{25}$) of *dextro*-rotatory derivatives[1] (8, 10, 42).

Compounds compared	Ethanol			Acetone			Acetic acid		
	amide	acid	anilide	amide	acid	anilide	amide	acid	anilide
Hydratropic acid (IX) . .	69.3°	119°	271°	70.1°	143°	216°	21.2°	131°	216°
Phenylbutyric acid (VIII)	92.3°	129°	253°	92.3°	168°	197°	43.9°	138°	219°
Phenylvaleric acid (VII) .	88.5°	114°	237°	88.2°	157°	184°	48.9°	128°	212°
Phenylcaproic acid (VI) .	80.1°	119°	222°	86.4°	158°	198°	55.8°	138°	210°
Phenyloenanthic acid (V)	95.6°	122°	229°	97.4°	160°	192°	80.3°	134°	221°

Table 4. Optical activities ($[M]_D^{25}$) of *dextro*-rotatory derivatives[1] (8, 10, 42).

Compounds compared	Ether			Benzene		
	acid	amide	anilide	acid	amide	anilide
Hydratropic acid (IX)	—	117°	344°	140°	137°	287°
Phenylbutyric acid (VIII) . . .	133°	163°	378°	157°	187°	327°
Phenylvaleric acid (VII)	118°	163°	363°	136°	172°	338°
Phenylcaproic acid (VI)	125°	156°	356°	144°	161°	330°
Phenyloenanthic acid (V)	124°	190°	361°	148°	191°	348°

[1] For convenience, the rotations have been assigned the *dextro*-compounds even if they are measured on their antipodes.

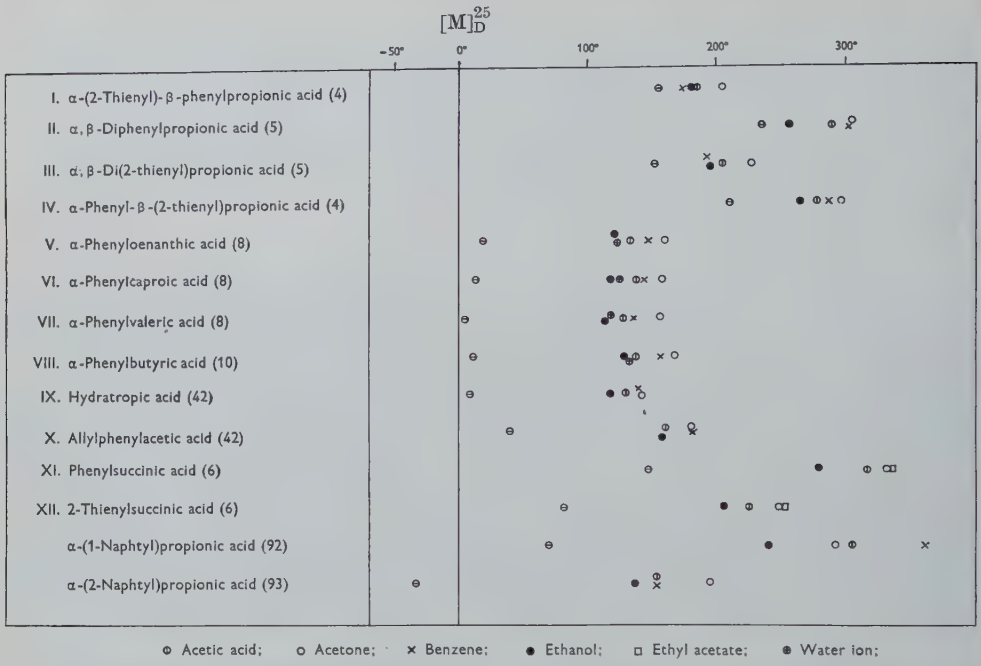


Fig. 9. Optical rotation ($[M]_D^{25}$) in different solvents of sterically related α-aryl carboxylic acids.

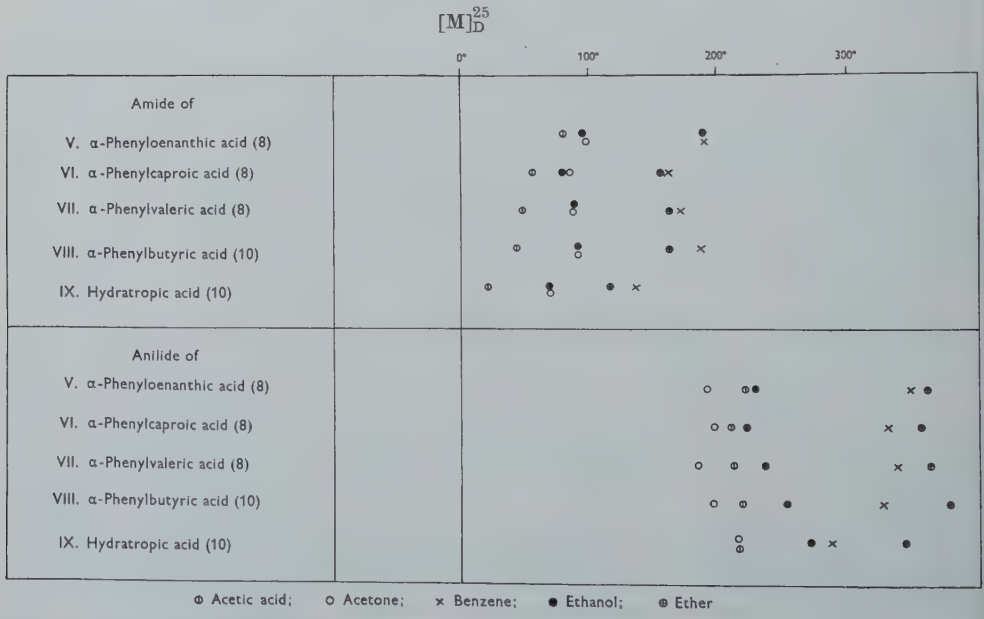


Fig. 10. Optical rotation ($[M]_D^{25}$) in different solvents of amides and anilides of sterically related α-phenyl fatty acids.

tive result but not a comparison between α-phenoxybutyric acid and α-(2-naphtoxy)butyric acid seems more difficult to explain (90). However, the special properties of the α-aryl carboxylic acids investigated allow some generalisations to be made.

In the paragraph dealing with the optical activities it was emphasized that the optical rotation of the α-aryl carboxylic acids mainly depends on the absorption bands of the α-aryl group, whereas the contribution from chromophores located at a distance from the asymmetric center is less important. An interaction of the solvent with the chromophores (a vicinal action from the solvent) should then be similar for compounds containing the same aryl group at the center. But the optical activity depends on the vicinal action of the remaining groups also. It is reasonable to suppose that a solvent action on these groups is mainly concerned with a strongly polar group. Therefore, compounds containing both a phenyl and a carboxyl group but no other chromophoric or polar group directly attached to the asymmetric center, would probably show a similar dependence on the solvent. The behaviour of a series of compounds is shown diagramatically in Figs. 9 and 10. All of the phenyl carboxylic acids have hydrogen as a common third group. The fourth group is, however, different, being arylmethyl (II, IV), alkyl (V–IX), allyl (X) and carboxymethyl (XI). In spite of these important structural differences, all of the acids show *exactly the same sequence of solvents*. It is rather probable that all α-phenyl carboxylic acids, which fulfil the requirements outlined above, may be sterically related by means of this method.

Comparison of the compounds with different α-aryl groups affords some interesting observations also. The α-(2-thienyl)propionic acids (I, III) differ from the phenyl analogs only in the position of the benzene dot (Fig. 9). It is evident that the significant parallellism between the behaviour of the related acids is a strong indication of the D-configuration of α,β-di(2-thienyl)propionic acid (III), which could not be related by other means. The arylsuccinic acids show the same sequence of solvents, but these acids were not measured in benzene. The values of α-(1-naphtyl)- and α-(2-napthyl)-propionic acids show that these compounds may also be included in the system.

The far-reaching similarities of the compounds with different α-aryl groups are in sharp contrast to the differences which occur, when the carboxyl group is replaced by amide or anilide groups (Fig. 10). However, the series of amides as well as the series of anilides each show a particular solvent sequence, clearly indicating the steric relation. This seems to indicate that the solvent acts predominantly on the polar carboxyl, amide and anilide groups.

Experimental

Catalytic reduction of α-phenyl fatty amides

(+)-*Hydratropamide:* 0.6 g of the (+)-amide was dissolved in dry ether and made up to 50 ml. The optical activity was measured in a 2 dm tube. $2\,\alpha_D^{23} = +1.9°$. The solution was added dropwise to a slurry of 0.2 g of lithium aluminium hydride in dry ether. When all the solution was added, the mixture was refluxed for half an hour and then 0.2 ml of water and 2 ml of 5 *N* sodium hydroxide were added successively. The pasty precipitate formed was extracted repeatedly with ether, and the ether fractions combined and dried. The optical activity was measured as before: $2\,\alpha_D^{23} = +0.4°$. The ether solution was treated with an excess of dry hydrochloric acid in

methanol and the mixture evaporated to dryness. The residue was extracted repeatedly with ether and finally recrystallized from a mixture of methanol and ether. The hydrochloride of α-phenylpropylamine was obtained as colourless needles, melting at 140–142°.

0.090 g of the chloride was dissolved in *ethanol* and made up to 10.0 ml: $2\,\alpha_D^{25} = -0.33°$, $[\alpha]_D^{25} = -18°$. 0.110 g in *water*: $2\,\alpha_D^{25} = -0.47°$, $[\alpha]_D^{25} = -21°$.

The hydrochloride was dissolved in water and the amine was liberated with 33 % sodium hydroxide and extracted with ether. The ether was removed and the residue evacuated at room temperature. 0.121 g of the remaining amine was dissolved in *ether*: $2\,\alpha_D^{25} = -0.39°$, $[\alpha]_D^{25} = -16°$. 0.136 g in *ethanol*: $2\,\alpha_D^{25} = -0.50°$, $[\alpha]_D^{25} = -18°$. 0.102 g in *75 % ethanol*: $2\,\alpha_D^{25} = -0.43°$, $[\alpha]_D^{25} = -21°$. Optical activity of the amine in the *homogenous state*: $0.5\,\alpha_D^{25} = -4.2°$, $[\alpha]_D^{25} = -8.4°$.

The ether washings, made before recrystallization of the hydrochloride, were combined and evaporated to 10 ml. The optical activity was measured: $2\,\alpha_D^{25} = +1.4°$.

(+)-α-*Phenylbutyramide:* 0.5 g of the (+)-amide was dissolved in 10 ml of dry ether: $2\,\alpha_D^{23} = +9.10°$. The hydrogenation was performed as described above. The ether washings, containing the amine, were combined and dried and the optical activity of the solution, which amounted to 25 ml, was measured: $2\,\alpha_D^{25} = +0.80°$. The amine was converted to hydrochloride as described above. The salt was obtained as colourless crystals, melting at 165–166°.

0.1033 g of the hydrochloride was dissolved in *ethanol* and made up to 10.0 ml and the activity measured: $2\,\alpha_D^{25} = -0.12°$, $[\alpha]_D^{25} = -5.8°$. 0.118 g in *water*: $2\,\alpha_D^{25} = -0.15°$, $[\alpha]_D^{25} = -6.4°$.

The amine was liberated and isolated as described above. 0.121 g in *ether*: $2\,\alpha_D^{25} = +0.25°$, $[\alpha]_D^{25} = +10°$. 0.119 g in *ethanol*: $2\,\alpha_D^{25} = -0.24°$, $[\alpha]_D^{25} = -10°$. 0.090 g in *75 % ethanol*: $2\,\alpha_D^{25} = -0.13°$, $[\alpha]_D^{25} = -7.2°$. Optical activity in the *homogenous state*: $0.5\,\alpha_D^{25} = +2.3°$, $[\alpha]_D^{25} = +4.6°$.

(+)-α-*Phenylvaleramide:* 1.2 g of the amide was dissolved in 50 ml of dry ether and the optical activity measured. $2\,\alpha_D^{25} = +4°$. The amide was hydrogenated as described above. The ether solution of the amine, which occupied 50 ml, was measured. $2\,\alpha_D^{25} = +0.63°$. The amine was converted to the hydrochloride, which was obtained as colourless needles melting at 208–210°.

0.110 g of the hydrochloride was dissolved in *water* and made up to 10.0 ml: $2\,\alpha_D^{25} = -0.15°$, $[\alpha]_D^{25} = -6.8°$.

The amine was liberated and isolated as described for the lower homologues. 0.137 g in *ether*: $2\,\alpha_D^{25} = -0.19°$, $[\alpha]_D^{25} = -6.9°$. 0.261 g in *ethanol*: $2\,\alpha_D^{25} = -0.46°$, $[\alpha]_D^{25} = -8.8°$. 0.196 g in *75 % ethanol*: $2\,\alpha_D^{25} = -0.27°$, $[\alpha]_D^{25} = -6.9°$. Optical activity in the *homogenous state*: $0.5\,\alpha_D^{25} = -2°$, $[\alpha]_D^{25} = -4°$.

To prevent loss of the material, the amines were recovered from the measured solutions by evaporation of the solvent. The amines were used without further purification for the measurements in other solvents. Therefore, the values obtained must not be considered as very accurate.

Melting-point diagrams

Melting-point preparations: Stock solutions of the two molecular compounds, obtained from the amides of hydratropic acid and α-phenylbutyric acid, and of racemic α-phenylbutyramide were prepared in acetone ("AnalaR" standards for laboratory chemicals). The desired mixtures were obtained by mixing appropriate

Table 5. Molecular compound of amides with opposite direction of rotation and racemic phenylbutyramide.

Mole % phenyl-butyramide	Melting point °C		
0	76	91.5	93
10			87 –89
20			84 –86.5
27	68		82.5–85
40	63.5		80.5–82.5
50	69.5		79.5–81.5
60	74		79 –80.5
70	77		79 –80
80	79.5		79 –80
85	80		–81
90	80		–82.5
100	80		–84

Table 6. Molecular compound of amides with the same direction of rotation and racemic phenylbutyramide.

Mole % phenyl-butyramide	Melting point °C	
0	66	70.5
10	64	68
20		65.5
30	60	66.5
40	57.5	70.5
50	67	73
60	71	77
70	75	78.5
80		61–80
90		61–82
100	80	84

amounts of the stock solutions, the solvent was removed and the residue carefully powdered and dried in a desiccator. The melting points were observed in a Kofler hot-stage microscope. The lower melting points, as shown by the dotted curves in Figs. 2 and 3, were obtained after premelting and rapid cooling of the samples under a cover-glass. Seeding of the samples was performed by scratching with a spatula dipped in the appropriate seeding material.

Summary

By means of experimental results reported in this and previous papers, a comparison between α-substituted phenyl and 2-thienyl carboxylic acids is made (Fig. 1, p. 299). The configurations of the compounds were related to D-glyceraldehyde.

The optical activities of the phenyl acids were throughout higher than those of the corresponding 2-thienyl compounds. It was assumed that the optical activity of the substituted 2-thienylacetic acids, in analogy with the corresponding phenyl derivatives, is mainly determined by the anisotropic bands of the aromatic group. The values of the optical activities indicate that the influence of chromophoric groups, separated from the asymmetric carbon atom by a methylene group, is less important. An indication of the optical purity of (−)-α,β-di(2-thienyl)propionic acid was emphasized.

The chemical and physical methods, used in the determination of steric relations, were briefly reviewed. In connection with the quasi-racemate method the conditions for racemate and quasi-racemate formation were discussed. Considerable differences in the ability for racemate formation were found between analogously constituted phenyl and 2-thienyl compounds.

The fact that (−)-α-phenylbutyramide gives molecular compounds with both (+)- and (−)-hydratropamide was discussed and the application of the quasi-racemate method in such cases demonstrated (Figs. 2 and 3, p. 310).

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Laboratory of Organic Chemistry, Chemical Institute, University of Uppsala, August 1956.

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Isolation and identification of a liver factor stimulating the incorporation of S^{35}-labelled sulphate into chondroitin sulphuric acid *in vitro*

By Lennart Rodén

With 2 figures in the text

The metabolism *in vitro* of the sulphate group of chondroitin sulphuric acid has been investigated by Boström and Månsson (1, 2), who used a system containing slices of costal cartilage from young calves. In a study of factors influencing the rate of incorporation of S^{35}-labelled sulphate into chondroitin sulphuric acid, it was found that the addition of a liver homogenate to the medium greatly increased the isotope uptake (3). The active principle was purified by fractional precipitation of a boiled liver extract with mercuric acetate, and paper chromatography resolved the "crude factor" thus obtained into a number of ninhydrin-positive spots, one of which contained all the stimulating activity (4). Observations reported in preliminary papers (5, 6) indicated that the active compound might be identical with glutamine. The present paper is an account of the isolation and identification of the liver factor as glutamine.

Experimental

Incorporation of S^{35}-labelled sulphate

Two methods have been used in the present investigation to follow the incorporation of isotope into chondroitin sulphuric acid in slices of cartilage.

Method I

The details of this method have been described earlier (2, 7). It involves isolation of the mucopolysaccharide from the slices after incubation, and determination of its radioactivity at infinite thickness. Analytic figures for three samples of chondroitin sulphuric acid prepared by this method are given in Table 1.

Method II

During the purification of the liver factor, a rapid method was evolved for assaying the stimulating activity (4). In this method, the radioactivity of the slices of cartilage was determined directly, after removal of the inorganic S^{35} sulphate by a special washing procedure.

The experimental error of the method is illustrated in Table 2, which shows the activity of 3 series of 10 separately incubated samples of cartilage obtained from the same piece of costal cartilage.

Table 1. Analytic values for 3 samples of chondroitin sulphuric acid (sodium salt) prepared according to Boström and Månsson (7).

	Per cent of dry weight of preparation			Theoretical values for $(C_{14}O_{14}H_{19}NSNa_2)n$ per cent
	1	2	3	
Galactosamine	25.0	25.4		35.59
	26.4	24.3		
Glucosamine	0.3	0.9		—
	0.6	0.7		
Hexuronic acid	39.5	40.6	40.1	38.57
Nitrogen	2.78	2.77	2.58	2.78
	2.77	2.78	2.60	
Sulphur	5.01	5.13	5.13	6.37
	4.94	5.30	5.14	
Ash	25.77	23.80	27.32	28.22
	25.08	22.55	27.63	

Table 2. S^{35} activity in slices of cartilage after incubation with S^{35}-labelled sulphate.

Each flask contained 4 slices of cartilage in 5 ml buffer (Krebs–Ringer bicarbonate in series 2 and Krebs–Ringer phosphate in series 1 and 3). After incubation at 37°C for 1 hour, 10 μC S^{35}-labelled sulphate in 1 ml buffer was added and incubation continued for 2 hours.

Sample no.	Series 1 c.p.m.	Series 2 c.p.m.	Series 3 c.p.m.
1	327	462	311
2	343	429	309
3	358	443	353
4	365	440	214
5	340	472	283
6	335	469	293
7	353	440	326
8	367	402	271
9	353	408	290
10	328	459	253
Mean value	347	442	290
Standard deviation .	$s_x = 14.5$	$s_x = 24$	$s_x = 39$

Nature of the S^{35} fixed in cartilage.—In the present study, the specificity of method II as a measure of sulpho-mucopolysaccharide synthesis was investigated with special attention to the possibility that S^{35} sulphate could be incorporated into sulphur-containing amino acids. To investigate this question the following experiments were carried out. 5 g of wet cartilage were cut into 0.3 mm thick slices and incubated at 37°C in 20 ml of Krebs–Ringer bicarbonate solution. After 0.5 hour, 250 μC of S^{35}-labelled sulphate in 5 ml of buffer solution was added, and incubation was continued for 7.5 hours. The material was then subjected to the usual washing procedure (4), which is known to remove all excess inorganic sulphate, boiled on a sand bath under reflux with 500 ml of 4 *N* hydrochloric acid for 25 hours, and filtered. The

Table 3. Nature of S^{35} activity fixed in cartilage after incubation with S^{35}-labelled sulphate.

For experimental details, see text below.

	c.p.m.
Activity in hydrolysate before passage through Dowex 2	134
Activity in hydrolysate after passage through Dowex 2	0
Recovery of S^{35}-labelled methionine added to the cartilage after incubation	
Activity added	59
Activity found in hydrolysate after passage through Dowex 2	48

filtrate was evaporated *in vacuo* to a small volume, and finally to dryness in a desiccator over sodium hydroxide. The material was then taken up in about 10 ml of water and kept overnight at −20°C. The insoluble material was again removed by filtration and washed with a few ml of water. One ml of the filtrate was diluted to 10 ml with water. Of this solution, 1 ml was plated on a 3.14 cm^2 lead planchet. The remainder was heated to boiling, and 1 ml of 10 per cent barium chloride solution was added. The resultant precipitate was removed by filtration through a Munktell OOH filter paper, and 1 ml of the filtrate was plated on a lead planchet. Before the addition of $BaCl_2$, 1965 counts/min. were found in the aliquot of the hydrolysate, and after precipitating the sulphate no activity could be detected in the filtrate.

In another experiment, two 5 g portions of cartilage were incubated as described above. After incubation and washing, 595,000 counts of S^{35}-labelled methionine (counted in a gas-flow counter at infinite thinness on aluminium plates) were added to one sample. Hydrolysis and the subsequent steps were then performed as above. The final filtrates were made up to 50 ml. The radioactivity was determined on 0.5 ml aliquots of a 100-fold diluted filtrate. 4 ml of each filtrate were passed through 2 × 25 cm columns of Dowex 2, 20–40 mesh, in the acetate form. The eluate was diluted to 400 ml and the radioactivity was again measured on 0.5 ml aliquots. As seen from Table 3, no activity could be detected after this treatment in the hydrolysate containing S^{35}-labelled sulphate only, whereas the hydrolysate containing S^{35}-labelled methionine as well retained about 80 per cent of the methionine activity added at the beginning of the experiment.

Consequently, the amount of S^{35} activity incorporated into the sulphur-containing amino acids must be negligible in comparison to that present as ester sulphate, and the S^{35} incorporation into whole cartilage studied in method II most likely represents a synthesis of chondroitin sulphuric acid.

S^{35} incorporation as a function of the sulphate concentration.—The sulphate concentration used in the previous studies (4) was that of the Krebs–Ringer bicarbonate solution prepared as described by Umbreit, Burris and Stauffer (8), i.e., 1.4×10^{-3} *M*. In one experiment the sulphate concentration was varied from 0.1×10^{-3} to 4.7×10^{-3} *M*. It is seen from Table 4 that the incorporation reached a plateau at about 0.9×10^{-3} *M*. Thus, the concentration already present in the Krebs–Ringer bicarbonate buffer is sufficient for the S^{35} uptake to proceed at a maximum rate. This concentration was therefore used without alteration.

Influence of buffer composition on S^{35} incorporation in normal and glutamine-stimulated cartilage.—Two different buffers have been used in the previous studies

Table 4. S^{35} incorporation into cartilage as a function of sulphate concentration.

Each flask contained 4 slices of cartilage in 5 ml sulphate-free Krebs–Ringer bicarbonate buffer + 1 ml S^{35}-labelled buffer containing in the different samples 0.1, 0.2, 0.4, 0.6, 0.8, 2.0 and 4.0 mg, respectively, of sodium sulphate with constant specific activity. Incubation at 37°C for 4 hours. The sulphate concentration of the usual sulphate-containing Krebs–Ringer bicarbonate buffer is 1.4 mM/l.

Sulphate concentration $M \times 10^{-3}$	Radioactivity c.p.m.
0.12	1531
0.24	1633
0.47	1703
0.70	1792
0.94	1874
1.17	1867
2.35	1787
4.69	1754

Table 5. Effect of buffer composition on S^{35} incorporation in normal and glutamine-stimulated cartilage.

Each flask contained 4 slices of cartilage in 5 ml sulphate-free buffer; sodium sulphate, 2 mg; glutamine when added, 100 μg. After incubation at 37°C for 1 hour, 1 ml carrier-free S^{35}-sulphate dissolved in sulphate-free buffer (1.6 mC/100 ml) was added and incubation continued for 2 hours.

	Radioactivity, c.p.m.		Stimulation by glutamine in per cent of control value
	Controls	Glutamine added	
Krebs–Ringer bicarbonate . . .	357	1324	371
Krebs–Ringer phosphate	290	523	180

(4) on S^{35} incorporation into slices of cartilage, namely, Krebs–Ringer bicarbonate solution and Krebs–Ringer phosphate solution (8). As reported by Boström and Månsson (2), no great difference exists between the S^{35} incorporation observed in the two cases. When Krebs–Ringer bicarbonate was used, the uptake was slightly higher, equalling 123 per cent of the incorporation in Krebs–Ringer phosphate solution (Table 5).

When glutamine was present in the medium, however, the composition of the buffer remarkably influenced the extent of stimulation. In Krebs–Ringer bicarbonate solution, the maximal stimulation generally reached about 370 per cent of the control value, whereas in Krebs–Ringer phosphate buffer the maximal uptake obtained under otherwise identical conditions was only about 180 per cent or less of the incorporation into the controls (Table 5). Krebs–Ringer bicarbonate solution was used in subsequent experiments.

Isolation of crystalline liver factor

For the isolation of larger amounts of the stimulating substance, it was decided to use large-scale preparative paper chromatography as described by Danielson and Hagdahl (9). The "crude factor" (4) was prepared from 3 kg of calf liver, the solu-

tion was evaporated *in vacuo* to about 7 ml, and absolute ethanol was added to a final concentration of 70 per cent. Two phases separated, and the solution was centrifuged in a small angle-centrifuge at about 1500 *g* for 5 minutes. The upper phase was then added to the top of a paper column, 33.0 × 11.5 cm, which had previously been washed with 70 per cent ethanol. The column was eluted with 70 per cent ethanol, and fractions of about 20 ml were collected at a rate not exceeding 100 ml/hour. The fractions were analyzed with the ninhydrin method of Moore and Stein (10). The conditions used permitted only partial separation of the active substance from the other ninhydrin-positive material. However, analytic paper chromatography in *sec*-butanol–formic acid–water (75:15:10) of fractions taken from various parts of the chromatogram revealed that a considerable enrichment of the active substance had occurred in its latter part. From the pooled fractions 161–181 and 182–221, 22.0 mg (sample A) and 28.8 mg (sample B) of substance could be crystallized by evaporating the solvent *in vacuo* to a few ml, adding ethanol until opalescence occurred, and leaving the solution in the refrigerator overnight. Sample A was recrystallized twice and sample B once from an ethanol-water mixture, yielding 10.4 mg and 18.0 mg, respectively. Nine mg of the latter sample was passed through a 1 × 6 cm column of Dowex 2 in the acetate form and recrystallized from ethanol–water.

Analytic methods

Glutamine was determined by the glutaminase method of Gale (11) as modified by Krebs[1] (12). Ammonia was determined as described by Conway (13). X-ray powder diffraction patterns of samples of glutamine and crystalline liver factor were taken in a Debye–Scherrer camera, using Cu K_α irradiation. Nitrogen determinations were carried out by the micro-Kjeldahl procedure (14). Ester sulphate was determined gravimetrically by precipitating as $BaSO_4$ after hydrolysis in 6 *N* hydrochloric acid for 4 hours.

Results

The identity of the liver factor with glutamine was indicated by the following facts.

1. *Melting point.* The crystalline liver factor samples A and B melted at 174°C and 183°C, respectively. Two recrystallizations increased the melting point of sample A to 181°C. After one recrystallization, sample B melted at 186–187°C. The latter value is in good conformity with the melting point of L-glutamine, 185–186°C (15). The recrystallized sample B, treated with Dowex 2, was used for the analyses described below.

2. *The nitrogen content* of the crystalline liver factor was 18.04 per cent (theoretical 19.17 per cent).

Fig. 1. X-ray powder diffraction patterns of L-glutamine (A) and crystalline liver factor (B).

[1] The *Clostridium welchii* strain SR 12 was kindly provided by Dr. D. A. Lowther. Different commercial samples of L-glutamine were used after one or two recrystallizations.

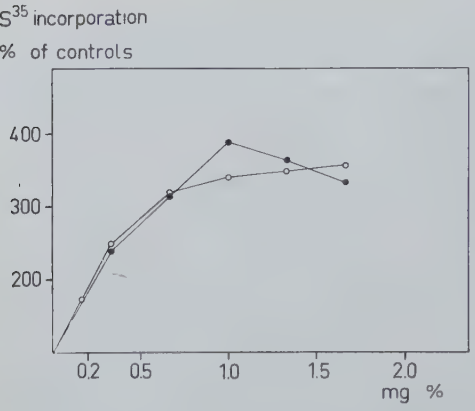

Fig. 2. S^{35} incorporation into cartilage in the presence of L-glutamine and crystalline liver factor. ○——○ Liver factor, ●——● L-glutamine. — Each flask contained 4 slices of cartilage in 5 ml Krebs–Ringer bicarbonate buffer; the addition of glutamine and liver factor ranged from 0–100 μg. After incubation at 37°C for 1 hour, 10 μC S^{35}-labelled sulphate in 1 ml buffer was added, and incubation continued for 2 hours.

Table 6. S^{35} incorporation into chondroitin sulphuric acid in the presence of glutamine.

Each flask contained 5 g of cartilage cut into 0.3 mm thick slices in 26 ml Krebs–Ringer bicarbonate solution; glutamine as indicated (0.2, 0.4, 0.6, 0.8 and 1.0 mg, respectively). After incubation at 37°C for 1 hour, 20 μC S^{35}-labelled sulphate in 2 ml buffer was added and incubation continued for 2 hours.

Concentration of glutamine $M \times 10^{-3}$	S^{35} incorporation c.p.m.	Per cent of control value
Experiment 1		
0	85 105 104	100
0.049	221 218	224
0.098	396 346	379
0.147	477 476	486
0.195	471 567	530
0.244	525	536
Experiment 2		
0	67 63	100
0.244	261 290	423

3. *The amide nitrogen content* as determined with the *Clostridium welchii* glutaminase method was found to be 9.33 per cent. An authentic specimen of L-glutamine yielded 9.01 per cent (theoretical 9.58 per cent).

4. *X-ray diffraction patterns* were identical for L-glutamine and the liver factor (Fig. 1).

5. *Paper chromatography* in three solvents: 80 per cent phenol; 2,4-lutidine saturated with water and *sec*-butanol–formic acid–water (75:15:10) showed only one ninhydrin-positive spot moving at the same rate as L-glutamine.

6. *Stimulation of S^{35} incorporation.* A comparison between the ability of L-glutamine and of the crystalline liver factor to stimulate the incorporation of S^{35}-labelled sulphate into cartilage (method II) showed that the two substances behaved identically within the limits of the method. It is seen from Fig. 2 that the S^{35} incorporation was doubled at a final glutamine concentration of 0.22 mg/100 ml (14.8×10^{-6} *M*). This figure was fairly constant and was reproduced several times. The stimulation reached a plateau level at an S^{35} uptake corresponding to 350–370 per cent of the incorporation into the controls.

Control experiments with method I showed that the increased S^{35} uptake observed by method II actually represented an increased incorporation into chondroitin sulphuric acid (Table 6).

Discussion

Although evidenced for a long time, the presence of glutamine in liver was only very recently proved by actual isolation (16). The isolation in a crystalline form and the analysis of the liver factor seem to establish with satisfactory certainty that it is identical with glutamine. The overall yield amounted to only 3 per cent, if it is assumed that the calf liver contains about 50 mg of glutamine per 100 g fresh tissue. It was reported earlier (4) that rat liver contained approximately 0.18 "S.E.S. units" per mg dry weight. One "S.E.S. unit" corresponds to 13 μg of glutamine. If the dry weight of the tissue is calculated to be 20 per cent of the wet weight, 100 g of fresh tissue thus contain about 3600 "S.E.S. units" corresponding to 47 mg of glutamine. This figure is in good agreement with the values reported in the literature for the glutamine content of rat liver (17), and all stimulating activity can thus be accounted for in terms of glutamine.

In studying the isotope incorporation into chondroitin sulphuric acid, method I, which involves isolation of the mucopolysaccharide in a fairly high degree of purity, provides accurate information. For several purposes, as for instance serial determinations of the influence of various factors on the isotope incorporation, the much more rapid method II can conveniently be employed. Especially when working with S^{35}-labelled sulphate, it can probably be assumed that the isotope is almost exclusively taken up by the chondroitin sulphuric acid. Earlier studies by a number of investigators (18–20) have failed to demonstrate any appreciable incorporation into sulphur-containing amino acids. Recently, however, Cremer and Dittmann (21) reported a comparatively high proportion of S^{35} incorporation *in vivo* into methionine and cystine, which were isolated by paper chromatography from a hydrolysate of rat cartilage after administration of S^{35}-labelled sulphate. No figures were given for the distribution ratio between amino acids and sulpho-mucopolysaccharide, but it seems that approximately $\frac{1}{10}$ of the activity reisolated from cartilage was found in the sulphur-containing amino acids. It was suggested by the authors that the discrepancy between their results and those of other workers could be due either to (1) selective incorporation of S^{35}-labelled sulphate into sulphur-containing amino acids in cartilage, bone and teeth, or (2) difference in age of the experimental animals. The present studies clearly demonstrate that incorporation of S^{35}-labelled sulphate into methionine *in vitro* is not a serious source of error in interpreting the sulphate uptake in cartilage as an expression of the synthesis of sulpho-mucopolysaccharide. The sulpholipids constitute another group of substances which could possibly incorporate S^{35}-sulphate (22) and thus interfere with conclusions regarding the metabolism of sulpho-mucopolysaccharides. So far, the presence of sulpholipids has not been demonstrated

in cartilage. In crucial experiments, the results obtained with method II have always been checked with method I, and the good conformity speaks in favour of the rapid method II in following the S^{35} incorporation into chondroitin sulphuric acid.

Summary

The incorporation of S^{35}-labelled sulphate into chondroitin sulphuric acid in slices of costal cartilage has been studied.

The S^{35} activity of chondroitin sulphuric acid was measured after actual isolation of the polysaccharide, or by direct measurement of the radioactivity of the slices after a special washing procedure, which removed inorganic S^{35}-labelled sulphate. The latter method was investigated with regard to its specificity as an expression of sulpho-mucopolysaccharide synthesis. It was found that S^{35} incorporation in sulphur-containing amino acids (methionine and cystine) did not constitute a source of error.

A factor present in liver homogenate, which stimulated the S^{35} uptake, has been isolated in crystalline form and identified as glutamine.

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Chemistry Department II, Karolinska Institutet, Stockholm, Sweden.

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Effect of glutamine on the synthesis of chondroitin sulphuric acid *in vitro*

By Lennart Rodén

With 7 figures in the text

Glutamine has recently been found to accelerate the incorporation of S^{35}-labelled sulphate into chondroitin sulphuric acid in surviving slices of cartilage (1). In the formation of hyaluronic acid by streptococci, the hexosamine nitrogen is derived from the amide group of glutamine, which has been suggested as an essential intermediate in the synthesis of hyaluronate glucosamine (2). Cell-free extracts of *Neurospora crassa* contain an enzyme system catalyzing the formation of glucosamine-6-phosphate from glucose-6-phosphate and glutamine (3).

In the light of the aforementioned investigations, it appeared highly probable that the effect of glutamine on the incorporation of S^{35}-labelled sulphate into chondroitin sulphuric acid was an indirect effect, mediated via the participation of glutamine in formation of the galactosamine moiety of chondroitin sulphuric acid. In an attempt to elucidate this question, the formation of the carbon skeleton of chondroitin sulphuric acid has been studied in the present work, using uniformly labelled C^{14}-glucose and 2-C^{14}-acetate. Further investigations of the effect of glutamine on the S^{35} uptake have also been pursued.

Experimental

Incorporation of C^{14}-labelled glucose and acetate

1. *Whole molecule*

Method I.—The methods for the study of S^{35} incorporation into chondroitin sulphuric acid have been dealt with in earlier papers (4–6). In the experiments with C^{14}-labelled glucose in the present work, method I (involving isolation of the polysaccharide) was used with the minor modification that dialysis was extended to 2 days. In order to examine the product for possible contamination with C^{14}-glucose from the incubation medium, it was subjected to the following procedure. The samples were dissolved in water, acidified to pH 1, and reprecipitated with ethanol. After dissolving once more in water, 20 mg of glucose were added, which represents a 100-fold excess over the amount of C^{14}-labelled glucose used in the experiment. The solution was centrifuged in a Servall centrifuge at about 17,000 *g* for 20 minutes, and precipitated with ethanol. The whole treatment produced no change in specific activity, and it was therefore omitted in subsequent preparations. The specific ac-

tivity of the chondroitin sulphuric acid was also determined as barium carbonate at infinite thickness after combustion.

METHOD II (in which the radioactivity of the slices of cartilage is measured directly) could also be used in the C^{14}-glucose experiments with the following modifications: (*a*) 20 per cent glucose was substituted for sodium sulphate in the washing procedure, and (*b*) the flasks containing the 20 per cent glucose solution were rocked in a water bath at 37°C, instead of the stationary incubation used earlier. Other details in the washing procedure were performed as described (6). Control slices, which had been treated with monoiodoacetic acid before incubation with C^{14}-labelled glucose, showed an incorporation not exceeding 2–4 per cent of that of the untreated slices. The results obtained with method II agreed reasonably well with the more accurate determinations afforded by method I.

2. *Uronic acid moiety*

The radioactivity of carbon atom 6 in the uronic acid moiety was determined after decarboxylation (7). 150 mg of C^{14}-labelled chondroitin sulphuric acid were hydrolyzed for 5 hours with 1 ml of 12 per cent hydrochloric acid at 111°C in a sealed glass bulb. The carbon dioxide evolved was precipitated in a manometric Van Slyke apparatus with 3 ml of a saturated barium hydroxide solution containing 10 per cent barium chloride. The precipitate was collected under ether, washed with carbon dioxide-free water and plated on a 1.1 cm^2 filter paper. The weight of two samples treated in this way was 37–38 mg; the radioactivity was thus measured under the conditions of infinite thickness.

3. *Hexosamine moiety*

To measure the specific activity of the amino sugar moiety, the rest of the hydrolysate used in the above experiment was decolorized with carbon, evaporated to dryness in a desiccator over sodium hydroxide, dissolved in a small quantity of water, and chromatographed on a 2×40 cm column of Dowex 50, according to Gardell (8). Half-hour fractions (8.5 ml) were collected and aliquots of 0.1 ml were taken for colorimetric analysis with the Elson–Morgan procedure (9). 0.5 ml aliquots from 35 fractions in the region where the glucosamine and galactosamine emerged were plated on lead planchets, and their radioactivity measured. The galactosamine-containing fractions were pooled, evaporated *in vacuo* to a small volume and, finally, to dryness in a desiccator over sodium hydroxide. The specific activity was subsequently determined as barium carbonate at infinite thickness after combustion.

4. *Acetyl group*

The incorporation of C^{14}-labelled acetate into chondroitin sulphuric acid *in vitro* has been reported by Boström and Månsson (10). Their method was used for the isolation of acetic acid. It involved refluxing of the chondroitin sulphuric acid with 10 per cent (v/v) H_2SO_4 on a boiling water bath and distillation of the acetic acid under atmospheric pressure. After neutralization to pH 7–8 with sodium hydroxide (glass electrode), the solution was evaporated in a rotary evaporator to dryness and, after combustion, the radioactivity of the barium carbonate planchets was measured at infinite thickness.

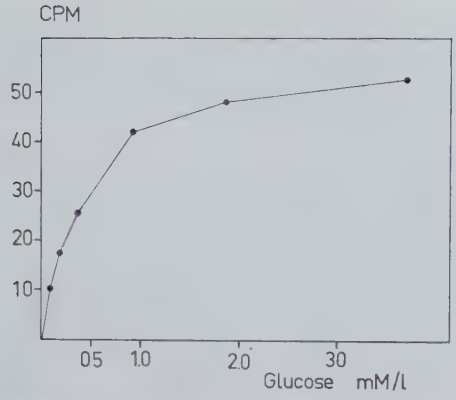

Fig. 1. Incorporation of C^{14}-labelled glucose into cartilage as a function of glucose concentration.

Each flask contained 4 slices of cartilage in 5 ml Krebs-Ringer bicarbonate buffer and C^{14}-labelled glucose (specific activity 0.2 μC/mg) in amounts ranging from 0.1 to 4.0 mg. The samples were incubated at 37°C for 2 hours.

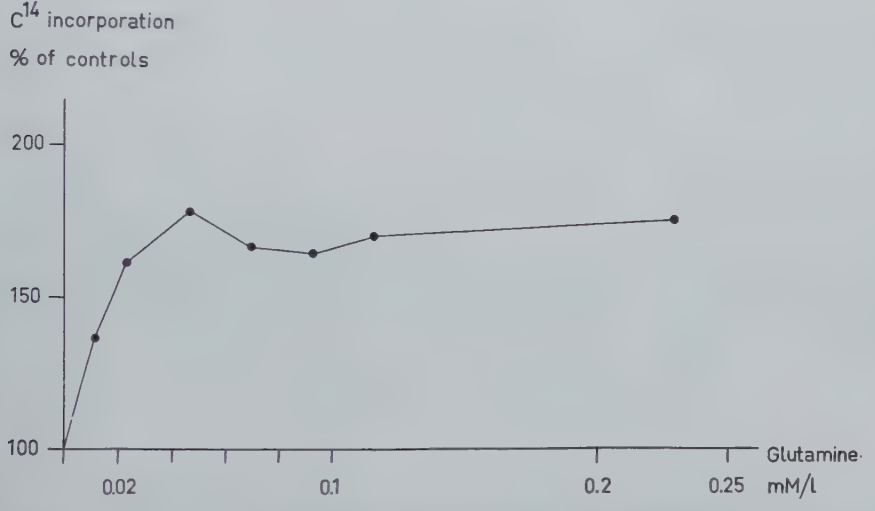

Fig. 2. Incorporation of C^{14}-labelled glucose into cartilage in the presence of glutamine.

Each flask contained 4 slices of cartilage in 5 ml Krebs–Ringer bicarbonate buffer and glutamine as indicated. 4 mg of C^{14}-labelled glucose (specific activity 0.125 μC/mg) was added after incubation at 37°C for 1.5 hour, and incubation was continued for 3 hours.

Results

A. *Incorporation of C^{14}-labelled glucose*

The rate of C^{14} incorporation as a function of the glucose concentration was determined using method II (Fig. 1). The influence of glutamine on the rate of C^{14}-glucose uptake was also studied, method II being used. An increased rate was observed amounting to a maximum of 180 per cent of the control value (Fig. 2).

To make certain that the C^{14} activity measured actually represented an incorporation into the chondroitin sulphuric acid, a similar experiment was carried out using method I. The glutamine concentration chosen was expected to give maximal stimulation. The results of this experiment are given in Table 1.

Table 1. Distribution of radioactivity in chondroitin sulphuric acid after incubation with C^{14}-labelled glucose and glutamine.

Each of two flasks contained 50 g of cartilage cut into 0.3 mm thick slices in 100 ml Krebs–Ringer bicarbonate solution. To one flask 10 mg glutamine was added. Incubation at 37°C for 2 hours. After addition of 20 μC C^{14}-labelled glucose (specific activity 115 μC/mg) in 2 ml buffer, incubation was continued for 3 hours. Chondroitin sulphuric acid was isolated and degraded as described under the Experimental section.

Compound	Carbon atom	Radioactivity*		
		Control c.p.m.	Glutamine added	
			c.p.m.	Per cent of control
Chondroitin sulphuric acid . . .	Entire molecule	63	141	224
Galactosamine	C_1–C_6	27	120	444
Uronic acid	C_6	104	190	183
N-acetyl	C_1–C_2	6.6	7.2	109

* Measured as $BaCO_3$ at infinite thickness.

The specific activity of the chondroitin sulphuric acid obtained from the slices incubated with glutamine was 223 per cent of the control value[1]. The polysaccharide was further degraded, as described under the Experimental section. In chromatographic separation of the amino sugar moiety (Fig. 3), the specific activity of the different galactosamine-containing fractions was fairly constant (Table 2), indicating

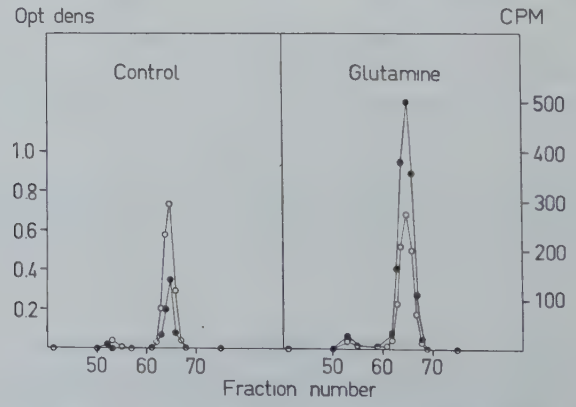

Fig. 3. Chromatographic separation of C^{14}-labelled amino sugars from normal and glutamine-stimulated cartilage. For further experimental details, see Table 1.

o——o Optical density at 535 mμ, •——• Radioactivity.

[1] Measured as chondroitin sulphuric acid at infinite thickness.

Table 2. Relative specific activity of chromatographically separated galactosamine-containing fractions from normal and glutamine-stimulated cartilage.

For experimental details, see Table 1.

Fraction number	Relative specific activity $\frac{\text{c.p.m.}}{\varepsilon_{535} \times 10^3}$	
	Control	Glutamine added
61	0	0.78
62	0.14	0.66
63	0.14	0.71
64	0.14	0.73
65	0.16	0.73
66	0.11	0.71
67	0	0.61
68	0	0.57

that the radioactivity was localized only to the amino sugar. The galactosamine of the glutamine-stimulated sample had a 4–5 times higher label than the control. A smaller increase was observed in the uronic acid moiety, amounting to slightly less than twice the control value. The label of the acetyl group was not significantly increased in the presence of glutamine.

Table 1 also shows the distribution of the radioactivity in the different parts of the molecule. In the control sample, more than $\frac{2}{3}$ were present in the uronic acidic moiety, whereas only about $\frac{1}{5}$ was localized to the galactosamine. A minor part of the activity was also found in the acetyl group.

A small quantity of glucosamine was present in the chondroitin sulphuric acid (Fig. 3), amounting to approximately 5 per cent of the total hexosamine. No accurate determination of the specific activity could be made, but it was fully evident that it had increased in the glucosamine derived from the glutamine-stimulated cartilage. This increase was not as large as that observed for the galactosamine.

B. *Incorporation of C^{14}-labelled acetate*

The uptake of C^{14}-labelled acetate was also accelerated in the presence of glutamine. In an experiment with method I, a glutamine concentration of 0.67×10^{-3} *M* increased the radioactivity of the chondroitin sulphuric acid more than ten times (Table 3).

C. *Incorporation of S^{35}-labelled sulphate*

Time curve for glutamine stimulation

The stimulation produced by glutamine was followed as a function of the incubation time, using method II. The isotope was added at the beginning of incubation, simultaneously with the glutamine. This experiment is recorded in Fig. 4. The slope of the control curve gradually decreased, whereas the glutamine curve showed an almost linear course. After 0.5 hour, the difference between the controls and the slices incubated with glutamine was small, but after 1 hour stimulation became fully evi-

Table 3. Incorporation of C^{14}-labelled acetate in chondroitin sulphuric acid in the presence of glutamine.

Each of two flasks contained 5 g of cartilage cut into 0.3 mm thick slices in 11 ml Krebs–Ringer bicarbonate solution; one flask contained 1 mg glutamine. After incubation at 37°C for 2 hours, 14.5 ml buffer containing 47×10^6 counts of sodium 2-C^{14} acetate (measured at infinite thinness in a windowless gas-flow counter) was added to each sample and another 1.5 mg glutamine to the glutamine-containing sample. Incubation was then continued for 3 hours.

	C^{14} activity* c.p.m.
Control	6 5
Glutamine added	72 66

* Measured as chondroitin sulphuric acid at infinite thickness.

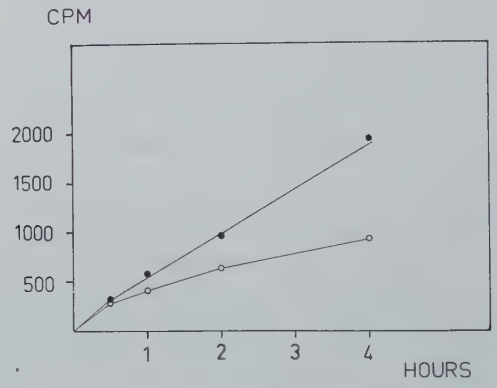

Fig. 4. Time curve for S^{35} incorporation into cartilage in the presence of glutamine. ○——○ Control, ●——●, L-glutamine.

Each flask contained 5 slices of cartilage in 5 ml Krebs–Ringer bicarbonate buffer; 10 μC S^{35}-labelled sulphate in 1 ml buffer; glutamine when added 50 μg.

dent. This finding is consistent with the results of other experiments, in which the slices were preincubated with glutamine for varying periods before addition of the isotope (Fig. 5). The maximum S^{35} incorporation in the presence of glutamine did not reach twice the control value, when preincubation was omitted or pursued for 0.5 hour. Prolongation of the preincubation period to 1 hour, followed by incubation with the isotope for 2 hours, increased the isotope uptake to almost 4 times the control level. Incubation of the samples for 2 hours before and 2 hours after addition of the isotope did not, however, raise the maximal S^{35} uptake any further. It must be pointed out in this connexion that, in the assay of stimulating activity, the slices were generally preincubated with the stimulating substance for 1 hour before addition of the isotope, incubation being continued for 2 hours thereafter.

In one experiment, glucose was present in the medium instead of glutamine. Labelled sulphate was added at the beginning of the experiment. The S^{35} incorpora-

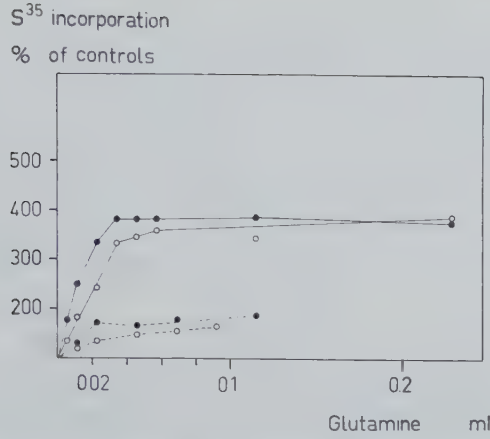

Fig. 5. S^{35} incorporation into cartilage in the presence of glutamine as a function of preincubation time before isotope addition.

o------o 0 + 1 hour, •------• 0.5 + 1 hour, o——o 1 + 2 hours, •——• 2 + 2 hours.

Each flask contained 4 slices of cartilage in 5 ml Krebs–Ringer bicarbonate buffer; 10 μC S^{35}-labelled sulphate in 1 ml buffer was added after incubation at 37°C for varying periods, and incubation continued as indicated above.

tion was determined after different incubation times and compared with controls. In this case the two curves did not diverge remarkably from each other, although the glucose-containing samples had a slightly higher uptake than the controls.

S^{35} uptake in the presence of glutamine and glucose

It was found earlier (6) that the active principle stimulating the S^{35} uptake in cartilage was present not only in liver, but also in homogenates of many other organs. Considerable activity was also present in serum. The substitution of serum for part of the Krebs–Ringer bicarbonate solution produced an increased rate of S^{35} uptake which approached 6 times that of the controls, when the serum/buffer quotient (v/v) was 1:5 (Fig. 6). This seemed surprising, when it was later found that such a high level of stimulation was not attainable even with pure glutamine. Consequently, it was necessary to assume the presence in serum of one or more active principles in addition to glutamine. Of the substances present in serum, glucose had already been shown to stimulate slightly the S^{35} incorporation into cartilage (6); moreover, it is known to be a precursor in mucopolysaccharide synthesis (11, 12). The effect of glucose in combination with glutamine on S^{35} incorporation was therefore tested in a number of experiments and some of the results are recorded in Table 4.

It was, in fact, found that an increase in S^{35} uptake invariably occurred which was greater than the sum of the stimulations caused by the two substances when tested separately in the same concentrations. The isotope incorporation nevertheless varied somewhat from one experiment to another. In most experiments, the S^{35} uptake in the presence of both glucose and glutamine rose to 400–500 per cent of the control value, but in a few cases it did not exceed 300 per cent. Even in these cases, however, the stimulation caused by a mixture of glutamine and glucose exceeded the sum of stimulations produced by the separate components.

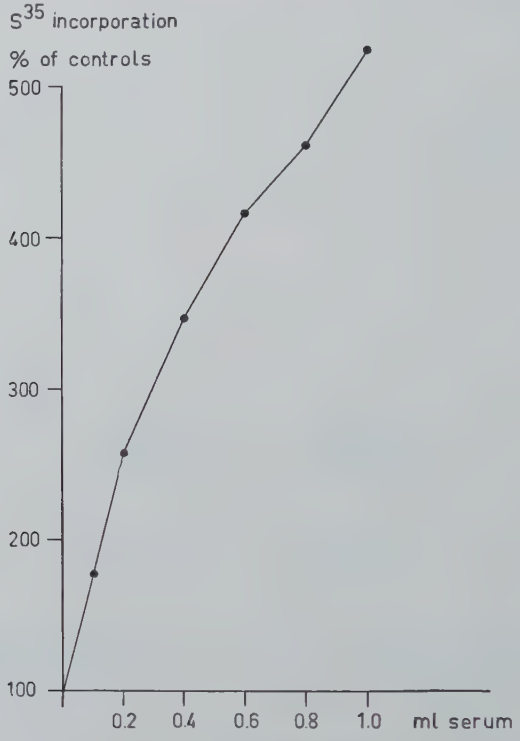

Fig. 6. S^{35} incorporation into cartilage in the presence of serum.

Each flask contained 4 slices of cartilage in Krebs–Ringer bicarbonate buffer with the addition of serum as indicated; total volume 5 ml. After incubation at 37°C for 1 hour, 10 μC S^{35}-labelled sulphate in 1 ml buffer was added, and incubation continued for 2 hours.

Table 4. S^{35} incorporation into cartilage in the presence of glucose and glutamine.

Each flask contained 4 slices of cartilage in 5 ml of Krebs–Ringer bicarbonate buffer. Additions were made as indicated. After incubation at 37°C for 1 hour, 10μ C S^{35}-labelled sulphate in 1 ml buffer was added, and incubation continued for 2 hours.

Substance	Amount mg	Radioactivity per cent of control value
Glucose	2	102
	4	101
	6	116
	8	118
	10	114
Glutamine	0.05	363
Glutamine 0.05 mg + glucose	2	431
	4	452
	6	424
	8	449
	10	422

Specificity for the stimulation of S^{35} incorporation

Asparagine was entirely ineffective in producing any stimulation of S^{35} incorporation. This also applied to ammonium ions alone or in combination with glutamate, and to glucose-6-phosphate. L-isoglutamine[1] produced slight stimulation, amounting to about 10 per cent of the glutamine effect. The material could be separated into two ninhydrin-positive components by paper electrophoresis in a 0.1 *M* bicarbonate buffer, pH 8.4 (13). The minor, slower-moving component migrated with the same velocity as glutamine and contained all stimulating activity. On the other hand, the major component, presumably isoglutamine, had no stimulating activity.

Discussion

Present concepts regarding the biosynthetic pathways in the formation of mucopolysaccharides are based partly on direct knowledge of the existence of enzymatic reactions, supposed to be involved in the synthetic route, and partly on scattered information about the presence in various tissues of substances which might play a role in the synthetic system, when bound together by postulated reactions. The evidence available has been reviewed recently by Dorfman (14) and by Calkins, Soodak and Bauer (15). The following scheme for the formation of chondroitin sulphuric acid (Fig. 7) is a slight modification of the scheme presented by the latter authors.

Special attention should be focused on the amino sugar formation. In a study of the formation of hyaluronic acid in a resting-cell suspension of group A streptococci, Lowther and Rogers (2) observed that the synthesis was dependent on the presence in the medium or in the cells of glutamine or other substrates (as ammonium glutamate) from which glutamine could be synthesized. Glutamine was therefore suggested as an essential intermediate in the formation of hyaluronic acid by streptococci. Leloir and Cardini (3) have shown that the mould *Neurospora crassa* contains an enzyme system which can form glucosamine-6-phosphate from glucose-6-phosphate and glutamine. Since isomerase was present in the crude enzyme preparation, the possibility could not be excluded that fructose-6-phosphate was, in fact, the substrate. After purification of the enzyme, Blumenthal *et al.* (16) concluded that fructose-6-phosphate was actually a closer precursor than glucose-6-phosphate.

It has been clearly demonstrated by the present investigations that glutamine does play a part in the biosynthesis of mammalian mucopolysaccharides as well. The role of glutamine as a nitrogen donor for the synthesis of glucosamine in microorganisms indicated a similar function in the formation of chondroitin sulphuric acid. This assumption was supported by the finding that the effect of glutamine was not confined to S^{35} incorporation. Increased rates of isotope uptake could be observed both when C^{14}-labelled acetate and C^{14}-labelled glucose were used as precursors. In the latter case, a large increase in label of the galactosamine moiety, as compared to the uronic acid, again indicated an intimate connexion between glutamine and hexosamine synthesis. As reported in a subsequent paper (17), glucosamine stimulated the S^{35} incorporation into chondroitin sulphuric acid to approximately the same maximal level as did glutamine. The simultaneous presence of the two substances did not cause an additional increase in isotope uptake.

[1] Kindly provided by Dr. Jesse P. Greenstein, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, U.S.A.

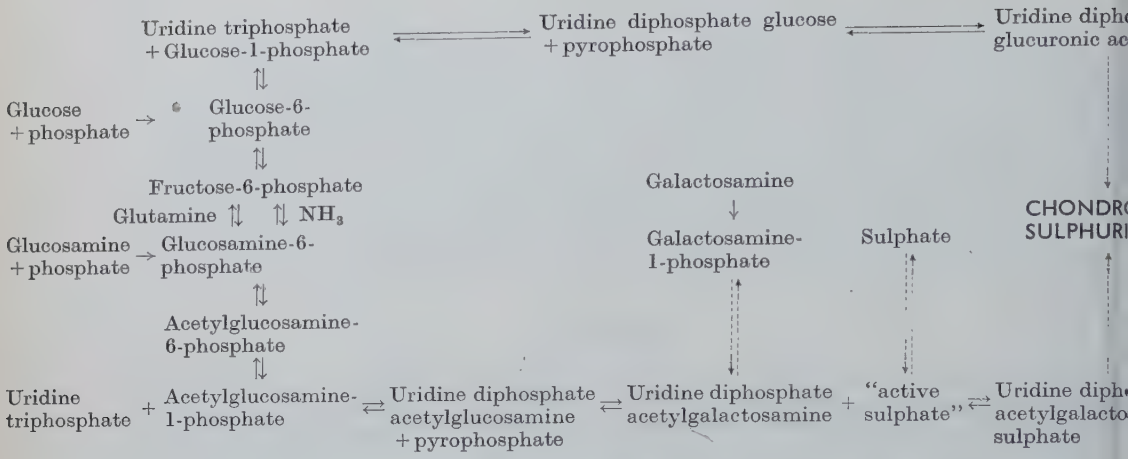

Fig. 7. Scheme of possible pathways for the biosynthesis of chondroitin sulphuric acid. Dotted lines indicate hypothetic reactions.

Although no conclusive data can be presented as to the site of involvement of glutamine in the reaction events, these results all strongly support the view that glutamine is primarily implicated in the amino-sugar producing reactions.

The increased label observed in the glucosamine contaminating the chondroitin sulphuric acid preparation, obtained after incubation of the cartilage with glutamine, also shows that the effect of glutamine is not confined to the formation of chondroitin sulphuric acid.

Additional evidence is available to make plausible the presence in animal tissues of enzyme systems catalyzing a hexosamine synthesis requiring glutamine. Castellani, Perri and Zambotti have claimed the presence in cartilage of an enzyme system catalyzing the glutamine-dependent synthesis of amino sugar (18). Pogell has demonstrated that rat liver contains a soluble enzyme catalyzing a reaction between glutamine and hexose-6-phosphate, to yield a product which in several respects behaves like glucosamine-6-phosphate (19).

Very recently, Leloir and Cardini demonstrated the presence in kidney tissue of an enzyme system catalyzing the conversion of acetylglucosamine-6-phosphate to fructose, ammonia and acetic acid (20). This reaction was reversible and thus afforded a new synthetic route to glucosamine, not involving glutamine.

In an *in vivo* study of the turnover rate of chondroitin sulphuric acid and hyaluronic acid in skin, Schiller *et al.* (21) found the C^{14} concentration to be the same in the whole mucopolysaccharide molecules and in the hexosamines, and from this inferred that the C^{14} concentration of the uronic acids was likewise identical. The chondroitin sulphuric acid isolated from cartilage in the present studies had a somewhat different distribution of the radioactivity. The ratio of the activity of the uronic acid to that of the amino sugar was 5:1 in the control samples, whereas the presence of glutamine decreased the ratio to about 1.5:1. It is possible that this difference depends, in part, on the use of an *in vitro* system.

From Fig. 4, showing the incorporation of S^{35}-labelled sulphate after varying periods of incubation, it is evident that a decrease in the rate of uptake with time took place in the absence of glutamine. This finding agrees with the earlier studies by

Boström and Månsson (22) and by Boyd and Neuman (23). It might indicate that a substance was consumed which could not be synthesized *in loco* at the same rate as it had been available under *in vivo* conditions. The fact that the curve was practically a straight line when glutamine was included in the medium might suggest that this substance was glutamine.

In the present work, it was found that glutamine was capable of producing a maximal increase in the rate of S^{35} incorporation into cartilage amounting to slightly less than 4 times the control value, and that glucose in combination with glutamine gave an S^{35} uptake approaching 5 times the control value. Substitution of serum for one-fifth of the buffer solution used in the incubations raised the uptake to almost 6 times. If allowance is made for the difference between the sulphate concentration in serum and in Krebs–Ringer-bicarbonate buffer (about 0.3×10^{-3} *M* and 1.4×10^{-3} *M*, respectively), which results in less dilution of S^{35} with non-labelled sulphate in the serum-containing specimens, the combined effect of glucose and glutamine is entirely sufficient to explain all stimulating activity present in serum.

The potentiating effect of glucose on the stimulation of S^{35} incorporation produced by glutamine is most easily explained by assuming that glucose becomes the rate-limiting substrate for the polysaccharide synthesis when glutamine is present in optimal amounts.

Summary

The effect of glutamine on the synthesis of chondroitin sulphuric acid in slices of cartilage has been studied.

It was found that C^{14}-glucose is incorporated in all three parts of the carbon skeleton: uronic acid, galactosamine and acetyl group. The specific activity of the uronic acid was about 4 times larger than that of the galactosamine. The label of the acetyl group was low in comparison with the rest of the molecule.

The addition of glutamine to the medium increased the label of the chondroitin sulphuric acid to more than twice the control value. The specific activity of the galactosamine and the uronic acid moiety increased 4–5 times and slightly less than twice, respectively.

The presence of glutamine in the medium also increased the incorporation of C^{14}-labelled acetate.

The stimulation of the incorporation of S^{35}-labelled sulphate by glutamine, which has been demonstrated earlier, was found to increase in the presence of glucose.

Preincubating the cartilage with glutamine before addition of S^{35}-labelled sulphate increased the maximal stimulation obtainable.

It is suggested that the increased utilization of the different isotopic precursors in the presence of glutamine results from a primary intervention in the hexosamine synthesis.

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Chemistry Department II, Karolinska Institutet, Stockholm, Sweden.

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Effect of hexosamines on the synthesis of chondroitin sulphuric acid *in vitro*

By Lennart Rodén

With 4 figures in the text

In the biosynthesis of hyaluronic acid in streptococci, glutamine is known to participate as a nitrogen donor for glucosamine formation (1). A similar function of glutamine in the synthesis of mammalian mucopolysaccharides was indicated by our studies on the formation of chondroitin sulphuric acid in slices of cartilage (2–4). Glutamine was found to be a rate-limiting substrate for the synthesis of mucopolysaccharide in this system, and an increased uptake of isotopic precursors took place when it was added to the medium. Since glutamine was most likely involved in the hexosamine synthesis, it seemed pertinent to investigate the influence of hexosamines on the formation of chondroitin sulphuric acid in the aforementioned *in vitro* system. The present paper describes the effects of glucosamine and galactosamine on the incorporation of S^{35}-labelled sulphate and uniformly labelled C^{14}-glucose into chondroitin sulphuric acid in slices of cartilage.

Experimental

Material

Glucosamine hydrochloride and galactosamine hydrochloride[1] were prepared from lobster shells and cartilage chondroitin sulphuric acid, respectively.

Methods

The *in vitro* methods for the study of isotope incorporation into chondroitin sulphuric acid in slices of cartilage have been described earlier (5, 6).

Results

A. *Incorporation of S^{35}-labelled sulphate*

S^{35} uptake in the presence of hexosamines

Both glucosamine and galactosamine (in the form of their hydrochlorides) were found to exert a stimulating effect on the rate of S^{35} incorporation (Figs. 1 and 2). The effect of glucosamine, however, extended far beyond that of galactosamine. The maximum level of stimulation obtained with glucosamine varied between 250 and 320 per cent of the control value in different experiments (method II), and was

[1] Both substances kindly provided by Dr. S. Gardell of this Institute.

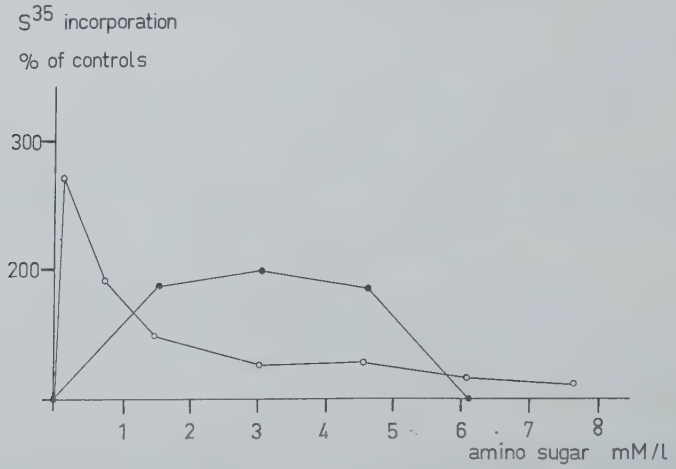

Fig. 1. S^{35} incorporation into cartilage in the presence of glucosamine HCl and galactosamine HCl. ○——○ Glucosamine HCl, ●——● Galactosamine HCl.

Each flask contained 4 slices of cartilage in 5 ml Krebs–Ringer bicarbonate buffer; addition of the hexosamine hydrochlorides ranged from 0.1 to 10 mg. After incubation at 37°C for 1 hour, 10 μC S^{35}-labelled sulphate in 1 ml buffer was added, and incubation continued for 2 hours.

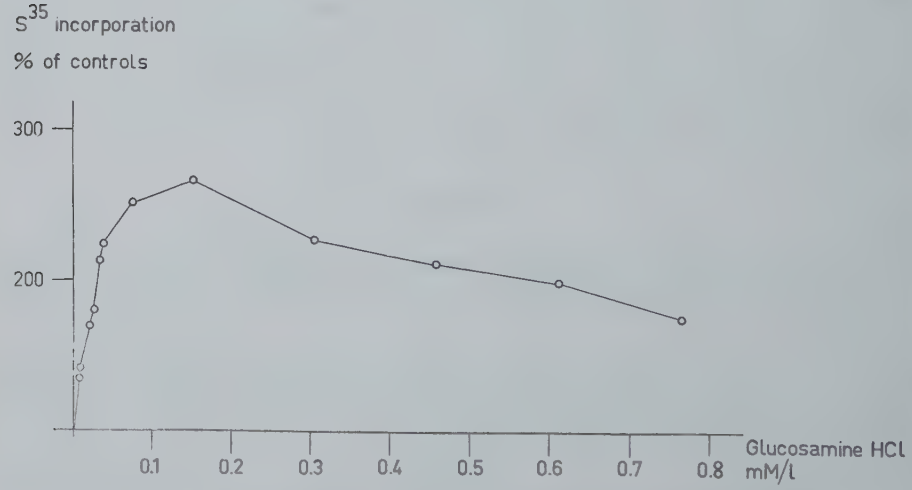

Fig. 2. S^{35} incorporation into cartilage in the presence of glucosamine HCl.

Each flask contained 4 slices of cartilage in 5 ml Krebs–Ringer bicarbonate buffer; addition of glucosamine HCl ranged from 10 to 1000 μg. After incubation at 37°C for 1 hour, 10 μC S^{35}-labelled sulphate in 1 ml buffer was added, and incubation continued for 2 hours.

reached at a glucosamine concentration of approximately 0.08×10^{-3} M. When the concentration of glucosamine was raised above 0.15×10^{-3} M, the stimulation gradually diminished, and only 140–170 per cent incorporation was observed at concentrations ranging from $3–6 \times 10^{-3}$ M.

Table 1. S^{35} incorporation into cartilage in the presence of glutamine and glucosamine.

Substance	Amount μg	Radioactivity per cent of control value
Glutamine	50	341
	100	328
Glucosamine	100	340
	200	334
Glutamine + glucosamine	50 + 100	362
	50 + 200	291
	100 + 100	314
	100 + 200	308

Each flask contained 4 slices of cartilage in 5 ml of Krebs–Ringer bicarbonate buffer. Additions were made as indicated. After incubation at 37°C for 1 hour, 10 μC S^{35}-labelled sulphate in 1 ml buffer was added, and incubation continued for 2 hours.

It is evident from a comparison between glutamine and glucosamine stimulation (ref. (4), and Fig. 2) that the glutamine curve rises more steeply to a plateau level. Whereas a glutamine concentration of 14.8×10^{-6} *M* was sufficient to produce a doubling of the S^{35} uptake, 30.9×10^{-6} *M* of glucosamine was necessary to give the same effect.

In the case of galactosamine, 3×10^{-3} *M* produced the maximum effect, amounting to nearly 200 per cent incorporation. At 6×10^{-3} *M*, the S^{35} incorporation had again fallen to the control value. Thus, in order to double the isotope incorporation, galactosamine had to be present in an amount approximately 100 times greater than that necessary for glucosamine.

When glutamine was present in amounts producing maximal stimulation, the addition of glucosamine did not further increase the S^{35} incorporation rate (Table 1).

S^{35} uptake in the presence of glucose and glucosamine

The influence of glucose on the stimulation of S^{35} incorporation by glucosamine was investigated in two sets of experiments (Table 2). The glucosamine concentration

Table 2. S^{35} incorporation into cartilage in the presence of glucose and glucosamine.

	Controls	Glucose	Glucosamine	Glucose + glucosamine
Radioactivity c.p.m.	184	219	520	266
	210	248	583	275
	196	278	536	305
	180	221	616	259
	179	247	639	258
Per cent of control value		128	305	144

Each flask contained 4 slices of cartilage in 5 ml Krebs–Ringer bicarbonate buffer. Additions of glucose and glucosamine hydrochloride were made as indicated to final concentrations of 4.6 and 0.077×10^{-3} *M*. After incubation at 37°C for 1 hour, 1 ml S^{35}-labelled buffer (approximate S^{35} content 5 μC/ml) was added and incubation continued for 2 hours.

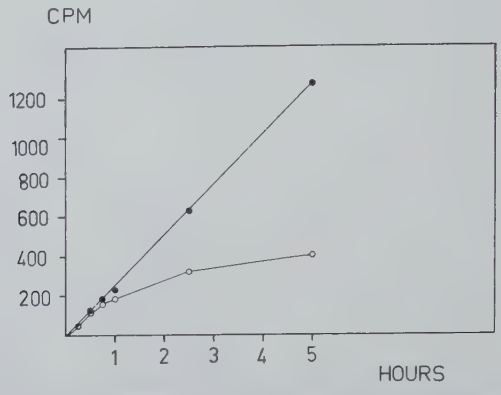

Fig. 3. Time curve for S^{35} incorporation into cartilage in the presence of glucosamine HCl. o——o Control, •——• Glucosamine HCl.

Each flask contained 4 slices of cartilage in 25 ml Krebs–Ringer bicarbonate buffer; 20 μC S^{35}-labelled sulphate in 2 ml buffer; glucosamine HCl when added 500 μg.

chosen caused a nearly maximal stimulation of S^{35} incorporation in the absence of glucose. The addition of glucose, however, lowered the S^{35} incorporation to a level slightly above that found when glucose alone was present in the medium in approximately physiologic concentration.

Time curve for glucosamine stimulation

The time curve for S^{35} incorporation in the presence of glucosamine (Fig. 3) showed the same general trend as the corresponding curve for glutamine (4). Whereas the slope of the control curve decreased after one hour, the glucosamine curve had approximately the same slope during the whole time period studied. The stimulation of the S^{35} incorporation by glucosamine did not become evident until after one hour.

B. *Incorporation of C^{14}-glucose in the presence of glucosamine*

The influence of glucosamine on the incorporation of C^{14}-glucose was studied in a number of experiments. With method II a small increase in C^{14} incorporation was observed at low glucosamine concentrations. When, however, the glucosamine concentration was raised, the C^{14} incorporation finally decreased to about 20 per cent of the original value. The S^{35} incorporation was determined parallelly in the same experimental series, and it is seen from Fig. 4 that the course of the S^{35} curve parallels that of the C^{14} curve, the two peaks of maximal stimulation appearing at the same glucosamine concentration. There are, however, two evident differences between the curves: (*a*) the stimulation of S^{35} incorporation greatly exceeds that of the C^{14} incorporation, and (*b*) whereas the C^{14} curve rapidly falls below the control value, the S^{35} incorporation remains slightly stimulated even at high glucosamine concentrations.

An experiment similar to that described above was carried out using method I. Two glucosamine concentrations were chosen; the lower one was expected to produce near-maximal stimulation of C^{14}-glucose incorporation, whereas the higher one was expected to decrease it considerably. Controls with S^{35}-labelled sulphate were run concomitantly. The isotope was added after 2 hours' preincubation, incubation then

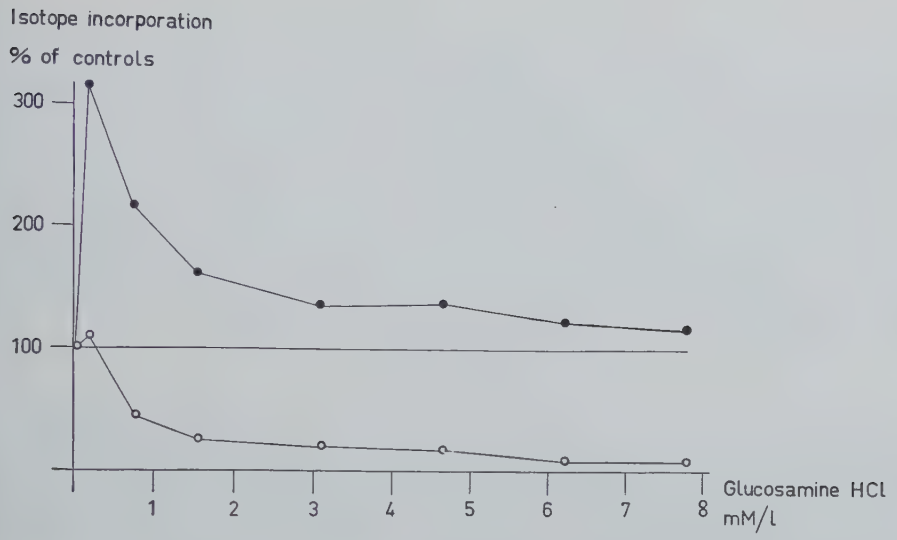

Fig. 4. Incorporation of S^{35}-labelled sulphate and C^{14}-labelled glucose into cartilage in the presence of glucosamine HCl. ●——● S^{35} activity, ○——○ C^{14} activity.

Each flask contained 4 slices of cartilage in 5 ml Krebs–Ringer bicarbonate buffer; glucosamine HCl as indicated. After incubation at 37°C for 2 hours, 10 μC S^{35}-labelled sulphate or 0.053 μC C^{14}-labelled glucose (specific activity 115 μC/mg), respectively, were added in 1 ml buffer. Incubation was then continued for 3 hours.

Table 3. Incorporation of radioactivity in chondroitin sulphuric acid from C^{14}-labelled glucose and S^{35}-labelled sulphate in the presence of glucosamine.

Compound	C^{14} activity of $BaCO_3$ at infinite thickness c.p.m.				S^{35} activity c.p.m.
	Chondroitin sulphuric acid	Galactos-amine	Uronic acid*	N-acetyl	Chondroitin sulphuric acid**
Control	146	43	222	7	136
	128	31	210	5	
Glucosamine 0.172×10^{-3} *M*	271	13	486	32	526
	293	9	456	30	
Glucosamine 8.60×10^{-3} *M*	59	0	85	7	354
	60		98	9	

* Activity of carbon atom 6.
** Measured directly at infinite thickness.

Each flask contained 10 g of cartilage cut into 0.3 mm thick slices in 26 ml Krebs–Ringer bicarbonate solution. Glucosamine HCl was added as indicated (1 mg and 50 mg, respectively). After incubation at 37°C for 2 hours, 7 μC C^{14}-labelled glucose (specific activity 115 μC/mg) or 10 μC S^{35}-labelled sulphate in 1 ml buffer was added, and incubation continued for 3 hours.

being continued for 3 hours. The result is seen in Table 3. In the presence of the lower glucosamine concentration, the S^{35} uptake was raised to 385 per cent of the control value, whereas at the higher concentration it was 260 per cent. The C^{14}-glucose incorporation was more than doubled at the low glucosamine concentration, whereas at the higher concentration it fell to about half the control value.

When the specific activities of the different parts of the molecule were compared, it was found that the amino sugar label was lowered at both glucosamine concentrations, whereas the label of the uronic acid was increased above the control value at the low glucosamine concentration and decreased below this value at the high concentration (Table 3). The label of the acetyl group was increased at the low glucosamine concentration and again lowered at the high concentration.

Discussion

It has been demonstrated that glucosamine can be incorporated into streptococcal hyaluronic acid without previous deamination and that it may, therefore, be an intermediate in the biosynthesis of this mucopolysaccharide (7). The degree of glucosamine utilization as compared to that of glucose was not, however, reported. Indirect evidence has been obtained in the present work for the transformation of glucosamine into the galactosamine of chondroitin sulphuric acid as well. It includes (*a*) stimulation of S^{35} incorporation by glucosamine together with (*b*) stimulation of C^{14}-glucose utilization for the synthesis of glucuronic acid and the simultaneous depression of the galactosamine label. The probable mechanism might involve a phosphorylation of the free amino sugar to glucosamine-6-phosphate, a process which was first shown by Harpur and Quastel (8) to take place in brain homogenates. After phosphorylation, the product lies on the main pathway from glucose to the galactosamine fixed in chondroitin sulphuric acid according to the scheme in (4). This scheme implies that the epimerization at carbon atom 4 in the glucose configuration to yield a galactose skeleton occurs in a bound form, represented in the scheme as a conversion of uridine diphosphate acetylglucosamine to uridine diphosphate acetylgalactosamine.

The low stimulation of S^{35} incorporation caused by galactosamine is explained if one assumes that this substance is a relatively inefficient precursor of chondroitin sulphuric acid galactosamine.

It is of interest to note that Lowther and Rogers (1) did not observe any stimulating effect of added glucosamine on the total synthesis of hyaluronate. In a surviving guinea pig colon, S^{35}-labelled sulphate was found to be incorporated into a dialyzable and a non-dialyzable fraction (9). The presence of glucosamine did not influence the rate of incorporation into either fraction, whereas glucose appreciably enhanced the uptake.

The phosphorylation of glucosamine by hexokinase is effectively inhibited by the presence of glucose. The Michaelis constant is 8×10^{-6} and 8×10^{-5} for glucose and glucosamine, respectively (10). The failure of glucosamine to produce more than a slight increase in the S^{35} incorporation when glucose was present can possibly be explained by a competition for hexokinase by glucose and glucosamine. Leloir (11) stated with respect to the phosphorylation of glucosamine that "it is difficult to decide whether this synthesis is a physiological process or simply an unspecific effect". It would appear from the above mentioned result that, under physiologic conditions, free amino sugars can participate in the biosynthesis of mucopolysaccharides only to a very limited extent. Actually, the presence of free amino sugars has not been

demonstrated in normal animal tissues (12, 13) except in one instance, namely, in cerebrospinal fluid (14).

A further point to be considered is the effect of glucosamine on the incorporation of C^{14}-glucose into the chondroitin sulphuric acid. In all experiments relating to this problem, the glucose concentration was kept low, so as not to inhibit the phosphorylation of glucosamine, which was assumed to be essential for any effect to be demonstrable. The following explanation seems to cover the experimental findings.

Case 1. Low concentration of glucosamine in medium.—Through the presumed phosphorylation process, glucosamine-6-phosphate was formed at a higher rate than could be achieved by the normal amination of hexose-6-phosphate in the controls. This resulted in an increased rate of formation of the mucopolysaccharide. Since the label in the uronic acid moiety predominated quantitatively, the total label of the molecule rose, whereas the galactosamine moiety acquired a decreased label due to dilution with a large pool of unlabelled precursor.

Case 2. High concentration of glucosamine in medium.—A decrease in label to less than half the control value was observed in the whole molecule as well as in the uronic acid moiety. The hexosamine had no measurable activity. This effect may be ascribed to either of two different causes: (1) diminished net synthesis of mucopolysaccharide below the control level; (2) besides the easily conceivable dilution of radioactivity brought about by the presumed transformation of glucosamine to glucosamine-6-phosphate, a similar dilution effect could be expected to occur in the formation of the uronic acid, if the glucosamine were in some way or other converted into intermediates lying on the pathway to the uronic acid as well.

In judging between the two hypotheses, the S^{35} uptake, which was followed in parallel experiments, provides information of possible value. It is highly probable that the S^{35} uptake is a reflection of the rate of synthesis of the whole mucopolysaccharide molecule and especially of its hexosamine moiety. Judging by such a criterion, alternative (1) does not seem very likely, as the S^{35} uptake at both glucosamine concentrations was well above the control value. The hypothesis discussed under (2) must thus be favoured. Although other possibilities cannot be ruled out, the results are consistent with the view that glucosamine entered the reaction sequences only by way of phosphorylation, and was transformed in the direction of galactosamine bound in chondroitin sulphuric acid at low concentrations, and towards glucuronic acid as well at the higher concentrations.

It remains to explain why the S^{35} incorporation at the higher concentration was considerably less than at the low concentration. At present, no more than a guess can be made, as there are too many unknown details in the steps involved in the synthesis of the mucopolysaccharide. It is possible, however, to explain the phenomenon in terms of known reactions. In the experiments under discussion, a preincubation period of 1–2 hours preceded addition of the isotope. During this time, a certain amount of glucosamine could have been transformed to glucosamine-6-phosphate and further to glucose-6-phosphate. It is well known that hexokinase is inhibited by glucose-6-phosphate (15), and if a considerable amount of this substance had accumulated, the phosphorylation of glucosamine would then have had to proceed at a slower rate than initially. As this reaction was probably rate-limiting for the synthesis of the mucopolysaccharide in the system used, the incorporation of S^{35}-labelled sulphate would then occur at a slower rate than at the lower glucosamine concentration. A relatively simple explanation is thus afforded for the course of the S^{35} incorporation curve in the presence of glucosamine in varying concentrations.

Summary

The participation of hexosamines in the synthesis of chondroitin sulphuric acid in slices of cartilage has been investigated. The following observations were made.

1. The incorporation of S^{35}-labelled sulphate in chondroitin sulphuric acid was enhanced by glucosamine to a maximum of about 3 times the control value. At high concentrations, the increase was smaller and approached the control value.

2. Galactosamine increased the S^{35} uptake to maximally twice the control value in a concentration 100 times larger than the glucosamine concentration which gave the same stimulation.

3. The maximal stimulation of S^{35} uptake by glucosamine could not be further increased by the addition of glutamine.

4. The stimulation of S^{35} uptake by glucosamine was almost completely abolished by glucose in physiologic concentrations.

5. The incorporation of C^{14}-labelled glucose into chondroitin sulphuric acid was increased at a low concentration of glucosamine and decreased at a high concentration in comparison with controls without glucosamine added. The label of the uronic acid was increased at the low glucosamine concentration and decreased at the high concentration. The galactosamine moiety of chondroitin sulphuric acid had a decreased label in both cases.

The implications of these findings are discussed.

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Chemistry Department II, Karolinska Institutet, Stockholm, Sweden.

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Communicated 6 June 1956 by Arne Fredga and Gunnar Blix

Quasi-racemic compounds between enanthiomorphs in the homologous series of 3-methyl-substituted long chain acids[1]

By Bo Hallgren

With 9 figures in the text

A fairly large number of optically active 3-methyl-substituted alkanoic acids has been prepared in this laboratory. It appeared of interest to examine binary systems of such acids in order to see if, or to what extent, quasi-racemic compounds are formed. Melting points and long crystal spacings are set out in Table 1. Details of the crystal structure of these acids are not yet available. Polymorphism has been reported in the case of (+)-3D-methyloctadecanoic acid (5) and (+)-3D-methyleicosanoic acid (8).

The enanthiomorphs of 3-methyltetracosanoic acid form a racemic compound melting at 68.6°. (−)-3L-Methyltetracosanoic acid forms a quasi-racemic compound with (+)-3D-methyltricosanoic acid. As shown in Fig. 1 this quasi-racemate has a melting point almost identical with that of 3DL-methyltetracosanoic acid. On rapid solidification of the melt a low-melting form is obtained. In this case the binary system has a eutectic point only. The melting point diagram of the binary system of (+)-3D-methyltricosanoic acid and (+)-3D-methyltetracosanoic acid also shows the existence of a eutectic point only (see Fig. 2).

The laevorotatory form of the next lower member of the series, (−)-3L-methyldocosanoic acid, forms, as shown in Fig. 3, a quasi-racemic compound with (+)-3D-methyltetracosanoic acid melting at 71.4°, that is higher than the racemic methyltetracosanoic acid. On the other hand the mixture of (−)-3L-methyldocosanoic acid and (−)-3L-methyltetracosanoic acid gives a melting point diagram with only one eutectic point (see Fig. 4).

In the third system studied in this work the difference in chain length is six carbon atoms. In this case, too, the quasi-racemic compound dominates the melting point diagram, as shown in Fig. 5 for the system composed of (+)-3D-methyloctadecanoic acid and (−)-3L-methyltetracosanoic acid. The melting point of the quasi-racemate is 66.0°. The mixtures of the enanthiomorphs with the same configuration give a melting point diagram with a eutectic point only (see Fig. 6).

Finally a system composed of 3-methyldodecanoic acid and 3-methyldocosanoic acid has been studied. In this case the components differ by ten carbon atoms and melt about 57 degrees apart. In spite of these differences (+)-3D-methyldodecanoic

[1] A preliminary account of this work was given at the meeting of the Swedish Biochemical Society at Upsala, Jan. 27–28, 1956.

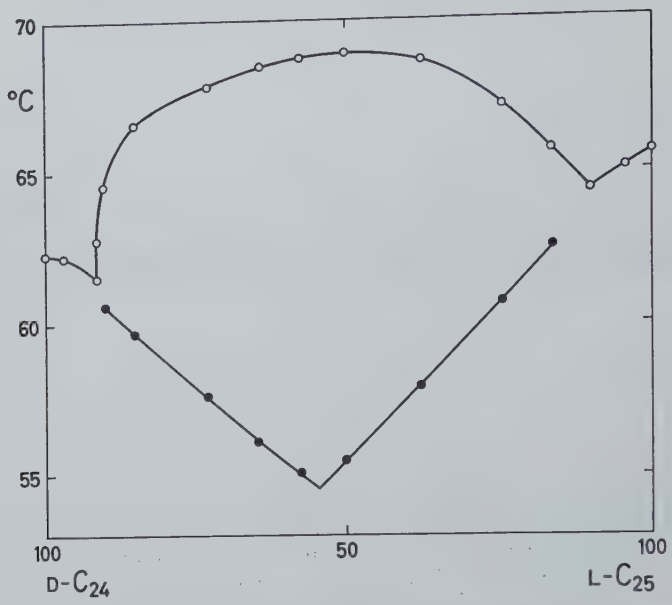

Fig. 1. Melting point diagrams (liquidus curves) of the binary system composed of (+)-3D-methyltricosanoic acid and (−)-3L-methyltetracosanoic acid.

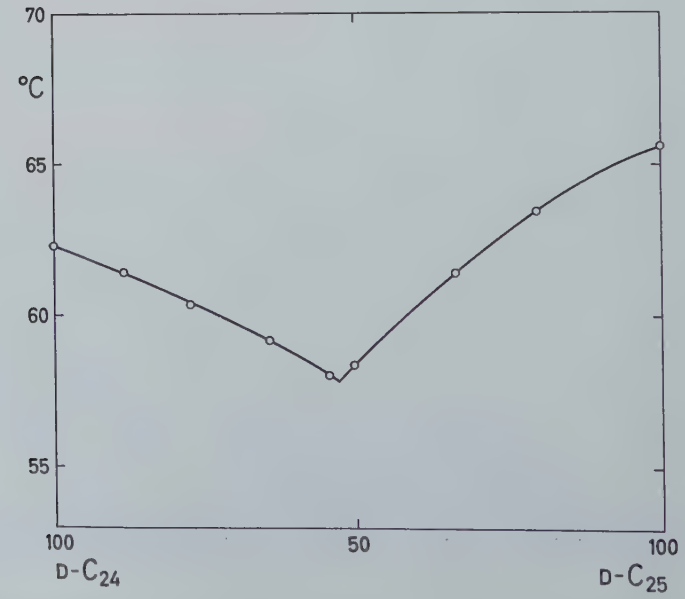

Fig. 2. Melting point diagram (liquidus curve) of the binary system composed of (+)-3D-methyltricosanoic acid and (+)-3D-methyltetracosanoic acid.

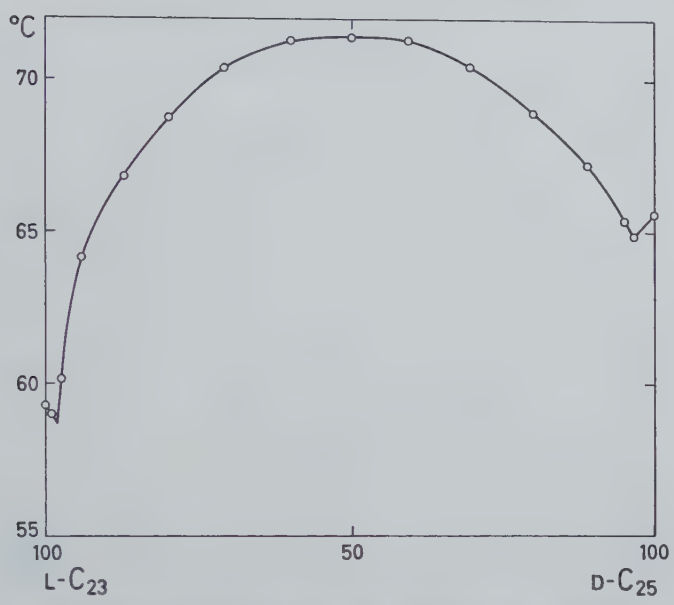

Fig. 3. Melting point diagram (liquidus curve) of the binary system composed of (−)-3L-methyldocosanoic acid and (+)-3D-methyltetracosanoic acid.

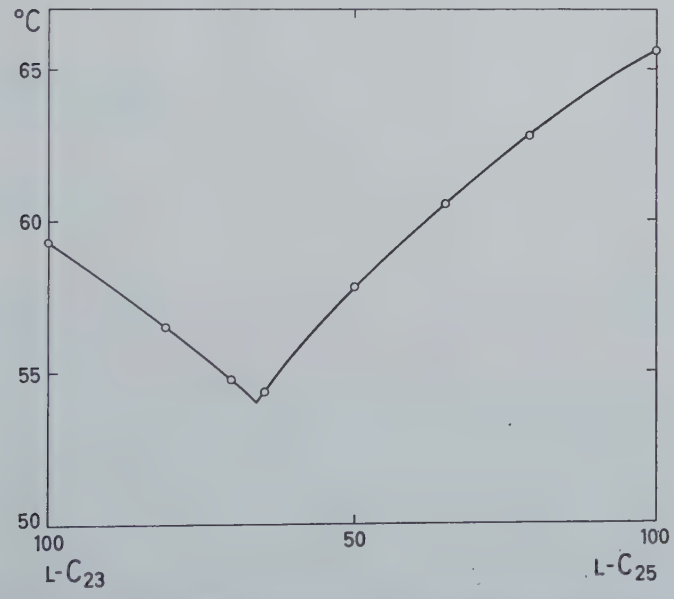

Fig. 4. Melting point diagram (liguidus curve) of the binary system composed of (−)-3L-methyldocosanoic acid and (−)-3L-methyltetracosanoic acid.

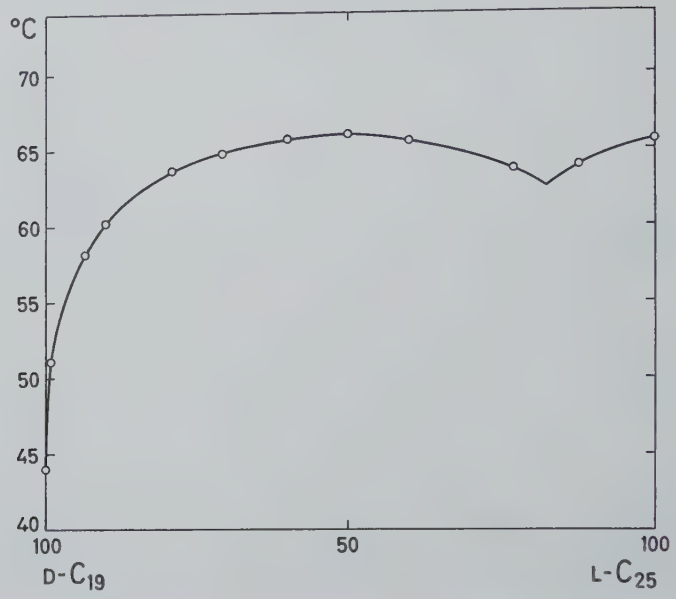

Fig. 5. Melting point diagram (liquidus curve) of the binary system composed of (+)-3D-methyloctadecanoic acid and (−)-3L-methyltetracosanoic acid.

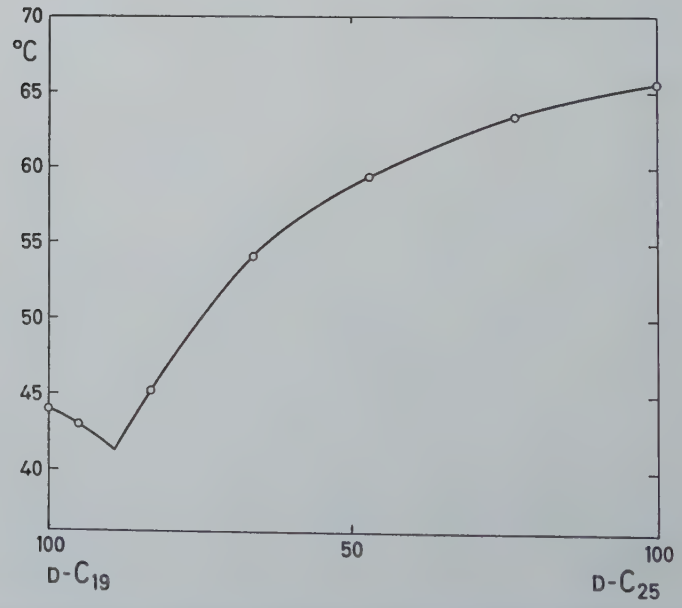

Fig. 6. Melting point diagram (liquidus curve) of the binary system composed of (+)-3D-methyloctadecanoic acid and (+)-3D-methyltetracosanoic acid.

Table 1.

Acid	Melting point, °C (References in parentheses)		X-Ray data, long spacings, Å (P = pressed specimen. References in parentheses)	
3DL-Methyldodecanoic acid	22	(1)		
(+)-3D-Methyldodecanoic acid	1.5–2.1	present investigation		
(−)-3L-Methyldodecanoic acid	1.7–2.3	present investigation		
3DL-Methyltetradecanoic acid	37	(1)		
	24–24.5	(2)		
3DL-Methylpentadecanoic acid	37.2–37.6	(3)		
3DL-Methylhexadecanoic acid	42.5–43	(2)		
3DL-Methyloctadecanoic acid	48.5–49	(4)		
	50.7–50.9	(5)	26.5	(5)
	50.8–51.3	(6)		
	51.0–51.5	(7)		
(+)-3D-Methyloctadecanoic acid	44.0–44.1		high-melting 25.9	(5)
	42.8–43.8	(5)	low-melting 36.6	(5)
(−)-3L-Methyloctadecanoic acid	43.9–44.1	(5)	25.9	(5)
(+)-3D-Methylnonadecanoic acid	47.8–48.2	(8)	33.2	(8)
(+)-3D-Methyleicosanoic acid	52.2–52.4	(8)	28.3 (8), after melting 28.3 and 35	(8) (8)
(−)-3L-Methyleicosanoic acid	52.2–52.5	unpublished data		
(−)-3L-Methyldocosanoic acid	59.1–59.3	present investigation*	30.6 P	(9)
3DL-Methyltricosanoic acid	63.5	(10)		
(+)-3D-Methyltricosanoic acid	62.1–62.3	(11)	32.3 P	(11)
3DL-Methyltetracosanoic acid	68.4–68.6	(12)	34.2 P	(12)
	68.0–69.1	(6)		
(+)-3D-Methyltetracosanoic acid	65.4–65.6	(12)	33.0	(12)
(−)-3L-Methyltetracosanoic acid	65.4–65.6	(12)	33.0	(12)
(+)-3D-Methylheptacosanoic acid	72.8–73.1	(13)	45.8 P	(13)

* Ställberg-Stenhagen (9) gives m.p. 58.1–58.3°. The same specimen has now repeatedly been found to melt exactly one degree higher. The cause of this discrepancy is uncertain. Polymorphism has not been observed.

acid and (−)-3L-methyldocosanoic acid form a sharply melting quasi-racemic compound. The binary melting point curve is reproduced in Fig. 7. In the right part of the diagram there seems to be a eutectic point at about 51°. Between 50 and 5 per cent of the higher-melting component the quasi-racemate shows a strong tendency to crystallize out both from the solvent and from the melt, leading to a tendency to record too high liquidus points in the left part of the diagram. The thaw point curve has been included in the diagram in spite of the well-known difficulties to determine correct thaw points. The corresponding curves for the system composed of (−)-3L-methyldodecanoic acid and (−)-3L-methyldocosanoic acid are shown in Fig. 8. There is no evidence for compound formation and no eutectic point is observed. In view of the great difference in molecular weight between the components and the less clear evidence for compound formation between components of opposite configuration it appeared desirable to check the behaviour of these systems by means of X-ray

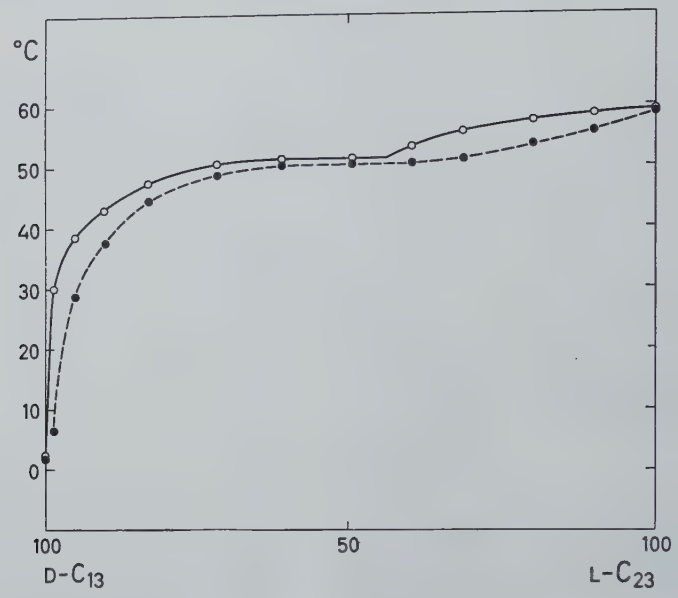

Fig. 7. Melting point diagram (liquidus curve) of the binary system composed of (+)-3D-methyldodecanoic acid and (−)-3L-methyldocosanoic acid. The dashed line represents the thaw point curve.

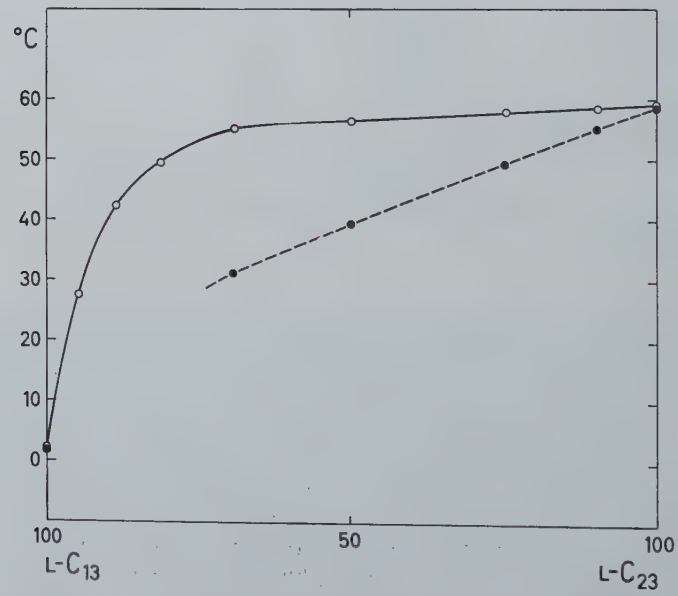

Fig. 8. Melting point diagram (liquidus curve) of the binary system composed of (−)-3L-methyldodecanoic acid and (−)-3L-methyldocosanoic acid. The dashed line represents the thaw point curve.

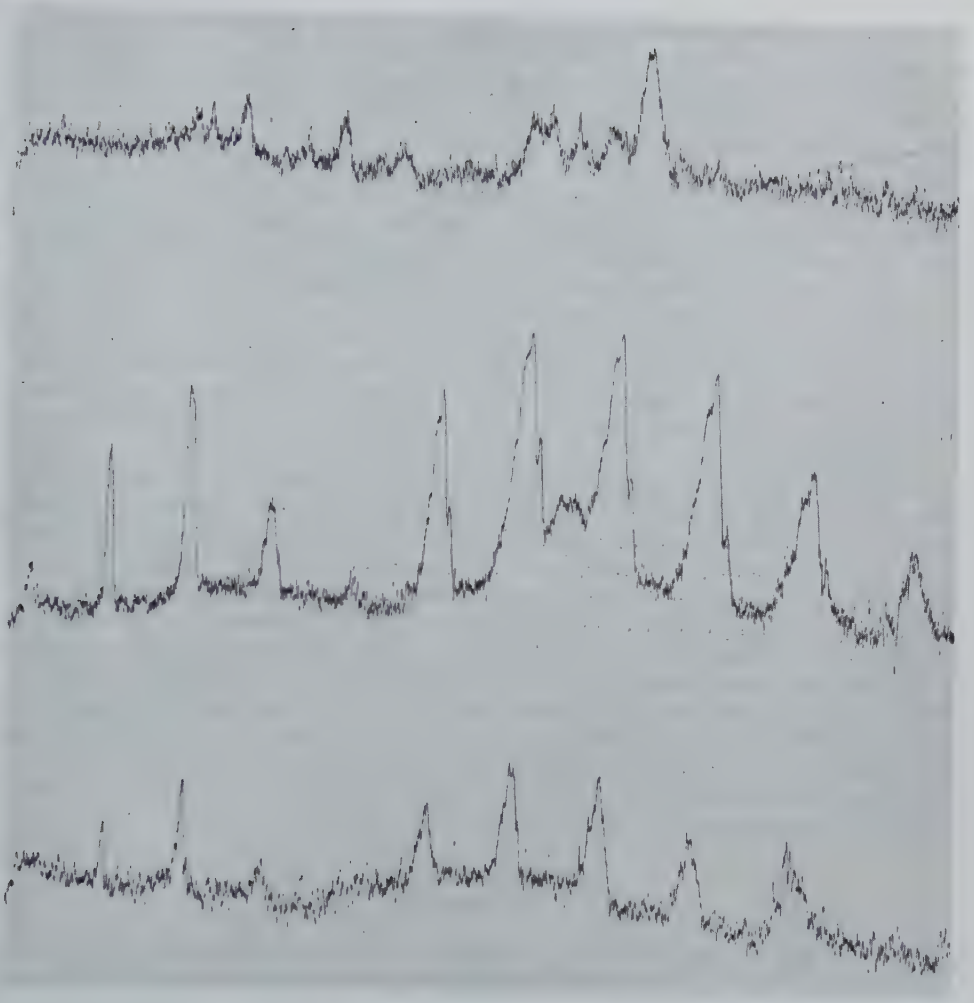

Fig. 9. Photometer tracings of X-ray diffraction patterns of an equimolecular mixture of (+)-3D-methyldodecanoic acid and (−)-3L-methyldocosanoic acid (upper trace), (−)-3L-methyldocosanoic acid (middle trace), and an equimolecular mixture of (−)-3L-methyldodecanoic acid and (−)-3L-methyldocosanoic acid (lower trace).

powder diffraction patterns. It was found that the quasi-racemate melting at 51.2° gave a diffraction pattern at room temperature which is quite different from that of the high-melting antipode, whereas the equimolecular mixture of the components having the same configuration was identical with that of the higher-melting component. Photometer tracings of the diffraction patterns are shown in Fig. 9.

Some systems have also been examined using the Kofler technique (14). The system composed of (−)-3L-methyldocosanoic acid and (+)-3D-methyltetracosanoic acid showed the expected behaviour. In the case of (−)-3L-methyleicosanoic acid and (+)-3D-methylheptacosanoic acid eutectic lines appeared at about 51° and

66° respectively. The intermediate part melted at the same temperature as the higher-melting component, but the behaviour of the system indicates that a quasi-racemic compound is formed also in this case. As could be expected from the appearance of the melting point diagram of Fig. 7 the Kofler technique did not reveal the quasi-racemate formed between (+)-3D-methyldodecanoic acid and (−)-3L-methyldocosanoic acid.

The existence of quasi-racemates that have a higher melting point than the true racemate of the highest melting component found in one of the systems now investigated does not appear to have been observed previously. Also quasi-racemates with such a big difference (corresponding to ten CH_2 groups) in the molecular weight between the components do not appear to have been described earlier. It is conceivable that systems composed of long chain fatty acids might easier allow such big differences between the components than systems composed of other types of molecules. It is interesting to note, however, that the steric configuration of the carbon atom carrying a small hydrocarbon side-chain outweighs the loss of ten carbon atoms in the main chain.

Lettré ((15, 16), cf. also Fredga (17)) has drawn parallels between the quasi-racemic compounds, which he called "partielle Razemate", and the compounds formed between enzymes and their substrates or between antigen and antibodies. It is well known from the field of immunological reactions that the specificity of precipitin reactions is not absolute. The same antibody can combine with antigens with somewhat different structures. In the present work it has been found that the length of the hydrocarbon chain is of considerably less importance than the stereochemical configuration at carbon atom 3. It is perhaps possible to draw a parallel between these phenomena and biological phenomena such as specific immunological precipitin reactions referred to above. In this case the polypeptide chains of the reacting proteins correspond to the hydrocarbon chains in our simple fatty acid systems.

The experimental technique used in the determination of the melting point diagrams has been described previously (18).

Summary

Several binary systems between optically active 3-methyl-alkanoic acids have been studied. It is found that stable quasi-racemates are formed between molecules having opposite configurations at carbon atom 3 even if the numbers of carbon atoms in the long chains differ by as much as 10 carbon atoms. No molecular compounds have been observed between acids that have the same steric configuration at carbon atom 3.

Department of Medical Biochemistry, Institute of Medical Chemistry, University of Uppsala.

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The complex formation between thallium(I) ions and halide, thiocyanate, and cyanide ions

By Rolf O. Nilsson

With 9 figures in the text

The complex formation between thallium and chloride ions has been studied by Hu and Scott [1], who determined the solubility of TlCl in chloride solutions. They found the species TlCl and $TlCl_2^-$ and confirmed the existence of them by spectrophotometric absorption measurements (Scott and Hu [2]). The absorption spectra of halide solutions saturated with thallium chloride, bromide and iodide were also studied by Fromherz and Lih [3], who ascribed the absorption bands to halocomplexes. The incomplete dissociation of TlCl and TlSCN has been studied by several investigators [4, 5, 6, 7, 8] by conductivity, emf and solubility measurements. The results are collected in Table 1. It can also be mentioned that Blayden and Davies [9] determined the solubility of TlCl in chloride solutions up to 0.2 *M* and assumed the presence of Tl^+ and TlCl.

In the present paper the complex formation was studied at 25°C and at the ionic strength 4 *M* (except in the fluoride system where the measurements were carried out at 1 *M*) with $NaClO_4$ as additional salt. The equilibrium constants, which thus are concentration constants, were determined by two methods, potentiometric titrations and solubility measurements. All measurements were carried out in neutral solutions. The dissociation constant of TlOH is 0.15 (Bell and George [4]) and indicates that the hydrolysis is negligible. From the equilibrium constant $[Tl^+]^3/[Tl^{3+}] \approx 10^{55}$ (calculated from standard potential data) and Benoit's [10] figures for thallic chloride and bromide complexes (for the chloride: $\beta_1 = 10^{8.1}$; $\beta_2 = 10^{13.6}$; for the bromide $\beta_1 = 10^{9.7}$; $\beta_2 = 10^{16.6}$) it follows that the thallic complexes present can be disregarded.

A. Potentiometric measurements

Theory

The measurements, which will be described in the following sections, show that only mononuclear complexes are formed in the solutions investigated, and the complex formation therefore can be written

$$Tl^+ + n\,A^- \rightleftharpoons TlA_n^{(n-1)-}. \tag{1}$$

The system is then defined by the complexity constants

$$\beta_n = [TlA_n^{(n-1)-}]/[Tl^+]\,[A^-]^n \qquad 1 \leqq n \leqq N \tag{2}$$

where *N* is the max. coordination number of Tl^+.

The total thallium content c_{Tl} is

$$c_{Tl} = [Tl^+]\left(1 + \sum_{n=1}^{N} \beta_n [A^-]^n\right) \tag{3}$$

or if $1 + \Sigma\beta_n[A^-]^n$ is called X (Fronaeus [11])

$$c_{Tl} = [Tl^+]\, X. \tag{4}$$

The total ligand ion concentration is

$$c_A = [A^-] + [Tl^+]\sum_{n=1}^{N} n\beta_n [A^-]^n. \tag{5}$$

The ligand number is defined (Bjerrum [12])

$$\bar{n} = \frac{c_A - [A^-]}{c_{Tl}}. \tag{6}$$

From (4), (5), and (6) we get

$$\bar{n} = \frac{\sum n\beta_n [A^-]^n}{X}. \tag{7}$$

The measurements were carried out as potentiometric titrations, at which $[Tl^+]$ was measured with a thallium amalgam electrode. In each series c_{Tl} was kept constant, while the ligand concentration was varied (*vide* Leden [13]). Emf for the following cell was measured

$$-\ \text{Tl, Hg} \;\Big|\; \text{S} \;\Big|\; \begin{matrix} 4\ M\ NaClO_4 \\ \text{or } 4\ M\ NaA \end{matrix} \;\Big|\; \text{RE} + \tag{8}$$

RE stands for the reference electrode (see under Apparatus). The emf of this cell is

$$E_m = E_0 - 59.16 \log [Tl^+] + E_j, \tag{9}$$

where E_j is the liquid junction potential and $E_0 = 1095.0$ mV (calculated from measurements in thallium perchlorate solutions). The solution S was obtained by mixing two solutions, one containing c_{Tl} mM $TlClO_4$ and $(4000 - c_{Tl})$ mM $NaClO_4$ and the other c_{Tl} mM $TlClO_4$ and $(4000 - c_{Tl})$ mM NaA. Nitrogen was passed through solution S during the measurements.

For the evaluation of the complexity constants the hypothetical cell

$$-\ \text{Tl, Hg} \;\Big|\; \text{S} \;\Big|\; 4\ M\ NaClO_4 \;\Big|\; \begin{matrix} c_{Tl}\ \text{m}M\ TlClO_4 \\ (4000 - c_{Tl})\ \text{m}M\ NaClO_4 \end{matrix} \;\Big|\; \text{Tl, Hg} + \tag{10}$$

was considered. The emf of this cell is

$$E = E_m - E_{m_0} = 59.16 \log \frac{c_{Tl}}{[Tl^+]} + E_j' = 59.16 \log X + E_j' \tag{11}$$

or

$$E' = E - E'_j = 59.16 \log X. \tag{12}$$

Table 1. Survey of investigations for $k_x = [TlA_{x+1}^{x-}]/[A^-]^x$.

Author	Investigated system	k_0 10^4 M	k_1 10^4	k_2 10^4 M^{-1}	k_3 10^4 M^{-2}	Temp. °C	Ionic strength	Method
Bell and George [4] . . .	SCN	10	—			25	zero	sol. of $TlIO_3$ in KA solutions
	Cl	9	—			25	zero	
Black and Garret [5] . . .	Cl	9				25	zero	emf
Garret and Vellinga [6] . .	Cl	8				25	zero	cond.
Onsager [7]	Cl	6				18	zero	,,
Davies [8]	Cl	6				18	zero	,,
Hu and Scott [1]	Cl	7.7	2.8			25	zero	sol. of TlCl in chloride sol.
This work	Cl	8	1.8	0.2		25	4 M	sol.
	SCN	10	7	2.5	1.1	25	4 M	emf and sol.

E_{m_0} denotes the emf of the cell (8) when S contains no halide ions. E_j' was calculated according to the approximate Henderson formula. The complexity constants were evaluated according to Fronaeus [11], i.e. the values of c_A are plotted against E' with c_M as parameter. The curves are then cut by straight lines for a series of values of E' and then it is possible to plot c_A as a function of c_M with E' as parameter. When these curves are extrapolated to $c_M = 0$, the ordinates give $[A^-]$. Corresponding values of X and $[A^-]$ are thus obtained. If no polynuclear complexes are formed the extrapolated curves should be straight lines with the slope $\bar{n}$. β_1 was obtained as the ordinate when $X_1 = (X-1)/[A^-]$ *versus* $[A^-]$ was extrapolated to $[A^-] = 0$. β_n was obtained in a similar fashion from $X_n = (X_{n-1} - \beta_{n-1})/[A^-]$ *vs.* $[A^-]$.

Apparatus

The *potentiometer apparatus* was from N. C. Jensen, Copenhagen, type 10A. A saturated Weston cell was used as the standard cell, which in turn was calibrated against two unsaturated Weston elements from The Eppley Lab. Inc.

The *galvanometer* was from *Norma Fabrik elektrischer Messgeräte G.m.b.H.*, Vienna, with a highest sensitivity of 2×10^{-9} A/mm.

The *nitrogen* passed through the solutions in the titration vessel during the emf measurements was taken from a bomb and led over copper filings at 600°C. The last 15 cm of the oven was held at a lower temperature. The gas was then passed through KOH solution, water and at last through 4 M $NaClO_4$ to avoid any evaporation in the titration vessel.

The reference electrodes used were saturated mercury(I) sulphate electrodes with 4 M $NaClO_4$ as ionic medium. Their emf's changed about 0.5 mV a month. All emf values in this paper are, for sake of simplicity, referred to the same E_0.

Chemicals used

Sodium perchlorate, hydrated (manufactured by G. F. Smith Chemical Co., Ohio) was used for making a chloride-free 4 M stock solution. The concentration was determined by percolating a diluted amount through a hydrogen-saturated cation exchanger and titrating the eluate with 100 mM NaOH.

Table 2. Potentiometric measurements in thallium perchlorate solutions.

c_{Tl} mM	E_m mV	E_j mV	E_0 mV
98.6	1,154.6	0.1	1,095.0
24.65	1,190.11	0.03	1,094.94
2.47	1,249.23		1,094.93
1.23	1,267.19		1,095.08
0.615	1,284.4		(1,094.5)
			Mean 1,095.0

Sodium chloride (Mercks p.a.), *sodium bromide* (Bakers analyzed), *sodium iodide* (Bakers analyzed), and *sodium thiocyanate* (Kebo puriss.) were used to make 4 M stock solutions. The concentrations were determined by the ion exchange method.

Sodium fluoride (Mercks p.a.) was dried and a 1 M stock solution was prepared by weighing.

Thallium perchlorate was made from Tl_2CO_3 (Mercks) and hot perchloric acid (Mercks p.a.). The precipitated thallium perchlorate was recrystallized three times from water.

The 55 % *thallium amalgam* used was made by fusing thallium (from Riedel-de Haën A.G., Hanover, and from The British Drug Houses Ltd., London) and Hg (Mercks p.a.) under paraffin. From the phase diagram for the Tl–Hg system (Kurnakow and Puschin [14]) it is clear that at 25°C the system consists of two phases, a liquid mercury–thallium phase and solid thallium (degenerated one-phase surface). Spencer [15] and Sucheni [16] have shown that the amalgam gives a constant potential value for thallium contents > 43 % by weight.

Measurements in thallium perchlorate solutions

To test that the thallium amalgam was suitable in perchlorate solutions, cell (8) was measured when S had the composition c_{Tl} mM $TlClO_4$ and (4000 − c_{Tl}) mM $NaClO_4$. The values in Table 2 show that the emf of the cell well follows Nernst's law.

The thallium fluoride system

On account of the low solubility of sodium fluoride the ionic strength had to be kept as low as 1 M. The following cell was measured

$$-\text{Tl, Hg} \mid \text{S} \mid 1\ M\ NaClO_4 \mid \text{RE}+$$

where S was obtained by adding a solution with the composition 100 mM $TlClO_4$, 550 mM NaF and 350 mM $NaClO_4$ to one containing 100 mM $TlClO_4$ and 900 mM $NaClO_4$. From these measurements no complex formation between thallium and fluoride ions could be detected. It may be mentioned that Bell and George [4] do not attach any importance to the value $\beta_1 \approx 1.2$ which they found for thallium fluoride. As is clear from the values in Table 3, E' decreases when the fluoride concentration increases. This can be due either to changing activity factors or to the presence of thallium perchlorate complexes. As the activity changes are unknown and as the liquid junction potential could be calculated only approximately no attempt was made to evaluate complexity constants for thallium perchlorate from the slight decrease in E'.

Table 3. Potentiometric measurements in the thallium fluoride system.
Ionic strength 1 *M*. $\beta_1 = 0$.

$c_{Tl} = 100$ m*M*			
c_{NaF} m*M*	*E* mV	E'_j mV	*E'* mV
96	−0.1	0.3	−0.4
184	−0.3	0.6	−0.9
277	−0.6	0.8	−1.4
415	−1.0	1.2	−2.2

The thallium chloride system

Cell (8) was measured. S was obtained by adding c_{Tl} m*M* $TlClO_4$, $4000 - c_{Tl}$ m*M* NaCl from a burette to c_{Tl} m*M* $TlClO_4$, $4000 - c_{Tl}$ m*M* $NaClO_4$ in the electrode vessel.

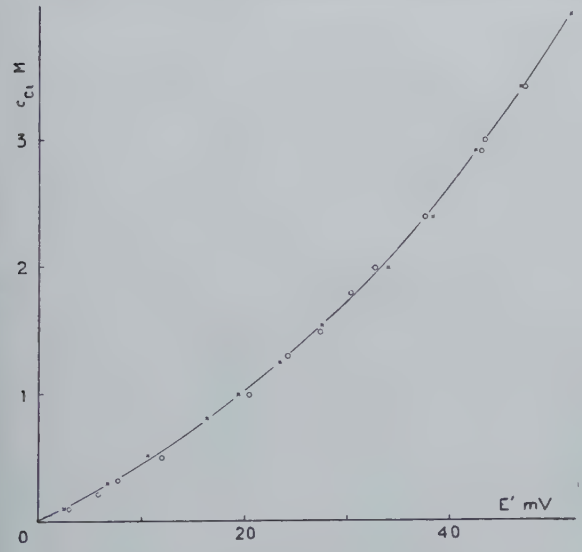

Fig. 1. c_{Cl} *vs.* *E'* for c_{Tl} 1 m*M* (O O O) and 2 m*M* (× × ×).

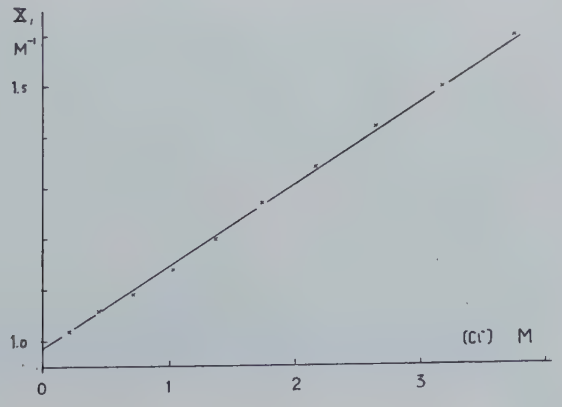

Fig. 2. X_1 *vs.* [Cl⁻]. $\beta_1 = 1.0\ M^{-1}$; $\beta_2 = 0.16\ M^{-2}$.

Table 4. Potentiometric measurements in the thallium chloride system.

$c_{Tl} = 1$ mM				$c_{Tl} = 2$ mM			
c_{Cl} M	E mV	E'_j mV	E' mV	c_{Cl} M	E mV	E'_j mV	E' mV
0.100	2.6	−0.1	2.7	0.100	2.4	−0.1	2.5
0.200	5.2	−0.1	5.3	0.300	6.6	−0.1	6.7
0.320	7.4	−0.2	7.6	0.500	10.4	−0.2	10.6
0.500	11.6	−0.2	11.8	0.810	15.9	−0.4	16.3
1.00	19.6	−0.4	20.0	1.00	18.8	−0.5	19.3
1.30	23.6	−0.6	24.2	1.25	22.8	−0.6	23.4
1.50	26.6	−0.7	27.3	1.54	26.8	−0.7	27.5
1.75	29.1	−0.8	29.9	2.00	33.1	−0.9	34.0
2.00	32.0	−0.9	32.9	2.40	37.1	−1.1	38.2
2.40	36.5	−1.1	37.6	2.93	41.3	−1.3	42.6
2.93	41.6	−1.3	42.9	3.43	45.5	−1.5	47.0
3.00	42.2	−1.3	43.5				
3.43	45.9	−1.5	47.4				
4.00	50.2	−1.7	51.9				

Table 5. Corresponding values of [Cl⁻], X, and $\bar{n}$.

$\beta_1 = 1.0\ M^{-1}$; $\beta_2 = 0.16\ M^{-2}$.

E' mV	[Cl⁻] mM	X	$\bar{n}_{calc.}$
5	210	1.22	0.18
10	450	1.48	0.35
15	720	1.78	0.50
20	1,030	2.18	0.63
25	1,370	2.65	0.74
30	1,740	3.21	0.85
35	2,160	3.90	0.93
40	2,640	4.74	1.03
45	3,180	5.76	1.11
50	3,760	7.00	1.19

On account of the low solubility of TlCl the thallium concentrations had to be chosen as low as 1 and 2 mM. From Fig. 1 it may be seen that the values belonging to the two series are lying within the limits of error on the same curve. This means that $c_{Cl} \approx [Cl^-]$. X and X_1 were calculated for a series of values of E', and X_1 was plotted against $[A^-]$ in Fig. 2. The straight line obtained gives $\beta_1 = 1.0\ M^{-1}$ and $\beta_2 = 0.16\ M^{-2}$. From these figures $\bar{n}$ is calculated according to eq. (7). The results are collected in Tables 4 and 5.

The bromide and iodide systems

Owing to the low solubility of TlBr and TlI, potentiometric measurements could not be carried out in the same fashion as in the previous systems. The complex formation was studied by solubility measurements as described in the later part of

this paper, but as it is impossible to judge from such measurements whether polynuclear complexes are formed or not [eq. (14), (15)], the following cell was measured.

$$-\text{Tl, Hg} \mid S_1 \mid \begin{matrix}\text{liquid}\\ \text{junction}\end{matrix} \mid S_2 \mid \text{Tl, Hg} +\,.$$

In the bromide system the liquid junction consisted of 7 *M* NaBr. S_1 and S_2 at the beginning contained 1 m*M* TlBr, 7 *M* NaBr. *E* was consequently 0. S_1 was then diluted with 7 *M* NaBr so that c_{Tl} dropped to 0.5 m*M*, while the bromide concentration was kept constant. *E* was measured to 17.8 mV, which is the theoretical value if only mononuclear complexes are formed [*vide* equations (3) and (9)]. Similar measurements were made on the iodide system, and here too only mononuclear species were found. In this case the liquid junction consisted of 8 *M* NaI, and S_2 contained 1 m*M* TlI, 8 *M* NaI.

The thiocyanate system

Three titration series were made in cell (8) with c_{Tl} 1, 5, and 13 m*M* respectively. In the first of these S was obtained by adding solution S_1 [c_{Tl} m*M* $TlClO_4$, $(4000 - c_{Tl})$ m*M* NaSCN] from a burette to solution S_2 [c_{Tl} m*M* $TlClO_4$, $(4000 - c_{Tl})$ m*M* $NaClO_4$] in the titration vessel. In the two latter series S_2 was added to S_1 on account of the low solubility of TlSCN. In these two series the salt bridge held 4 *M* NaSCN. The *E'* values are shown in Table 6 and Fig. 3 and the *X* values are given in Table 7.

Table 6. Potentiometric measurements in the thiocyanate system.

c_{Tl} = 1 m*M*				c_{Tl} = 5 m*M*				c_{Tl} = 13 m*M*			
c_{SCN} *M*	*E* mV	E_j' mV	*E'* mV	c_{SCN} *M*	*E* mV	E_j' mV	*E'* mV	c_{SCN} *M*	*E* mV	E_j' mV	*E'* mV
0.13	4.5		4.5	4.00	111.5	0.4	111.1	3.99	111.4	0.4	111.0
0.25	8.9		8.9	3.93	110.2	0.4	109.8				
0.36	13.1		13.1	3.87	108.9	0.4	108.5				
0.47	16.9		16.9	3.81	107.7	0.4	107.3				
0.84	29.8	0.1	29.7	3.75	106.5	0.4	106.1				
1.14	39.6	0.1	39.5	3.64	104.1	0.4	103.7				
1.39	47.4	0.1	47.3	3.53	101.9	0.4	101.5				
1.60	53.6	0.2	53.4	3.43	99.8	0.3	99.5				
1.78	58.9	0.2	58.7	3.33	97.8	0.3	97.5				
1.93	63.3	0.2	63.1	3.16	93.8	0.3	93.5				
2.06	66.8	0.2	66.6	3.00	90.2	0.3	89.9				
2.18	70.0	0.2	69.8	2.86	86.9	0.3	86.6				
2.39	75.6	0.2	75.4	2.73	83.8	0.3	83.5				
2.49	78.0	0.3	77.7	2.61	80.8	0.3	80.5				
2.57	80.0	0.3	79.7	2.50	78.2	0.2	78.0				
2.75	84.4	0.3	84.1	2.40	75.6	0.2	75.4				
2.88	87.4	0.3	87.1	2.31	73.3	0.2	73.1				
3.01	90.0	0.3	89.7	2.22	71.0	0.2	70.8				
3.14	93.7	0.3	93.4	2.14	68.9	0.2	68.7				
3.27	96.5	0.3	96.2	2.07	66.9	0.2	66.7				

Table 7. Corresponding values of [SCN⁻], X, and $\bar{n}$ in the thiocyanate system.

$\beta_1 = 1.4\ M^{-1}$; $\beta_2 = 1.0\ M^{-2}$; $\beta_3 = 0.35\ M^{-3}$; $\beta_4 = 0.12\ M^{-4}$.

E' mV	[SCN⁻] M	X	$\bar{n}_{calc.}$
5	0.135	1.21	0.19
10	0.280	1.48	0.38
15	0.410	1.79	0.55
20	0.560	2.18	0.76
25	0.700	2.65	0.92
30	0.850	3.21	1.10
35	1.00	3.90	1.26
40	1.16	4.74	1.42
45	1.32	5.76	1.58
50	1.48	7.00	1.74
55	1.65	8.51	1.88
60	1.82	10.3	2.02
65	2.01	12.5	2.18
70	2.20	15.2	2.31
75	2.39	18.5	2.42
80	2.59	22.5	2.53
85	2.79	27.4	2.63
90	3.01	33.2	2.71
95	3.23	40.4	2.80
100	3.46	49.0	2.88
105	3.70	59.6	2.94
110	3.95	72.3	3.03

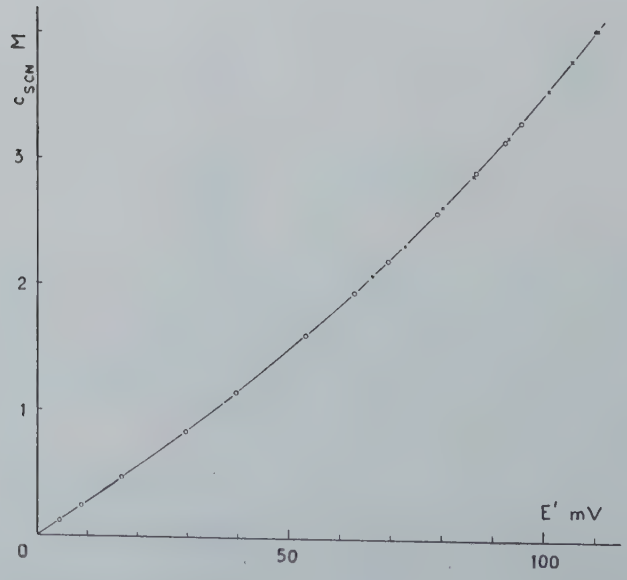

Fig. 3. c_{SCN} *vs.* E' for c_{Tl} 1 mM (○ ○ ○), 5 mM (× × ×), and 13 mM (△ △ △).

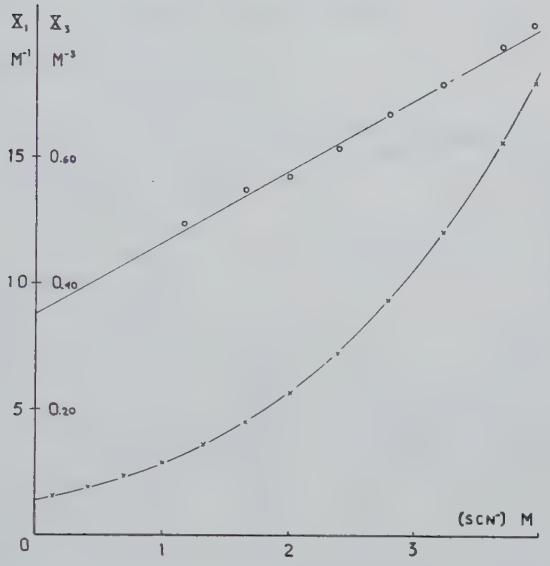

Fig. 4. X_1 ($\times$ $\times$ $\times$) and X_3 (O O O) *vs.* [SCN$^-$]. The drawn lines calculated with the constants $\beta_1 = 1.4\ M^{-1}$, $\beta_2 = 1.0\ M^{-2}$, $\beta_3 = 0.35\ M^{-3}$, $\beta_4 = 0.12\ M^{-4}$.

The functions X_1, X_2, and X_3 plotted against [A$^-$] gave $\beta_1 = 1.4\ M^{-1}$, $\beta_2 = 1.0\ M^{-2}$, $\beta_3 = 0.35\ M^{-3}$, and $\beta_4 = 0.12\ M^{-4}$. The $\bar{n}$ values in Table 7 are calculated from these figures according to eq. (7). X_1 and X_3 *vs.* [A$^-$] is shown in Fig. 4.

The cyanide system

Cell (8) was measured and S was prepared by adding a solution with the composition c_{Tl} mM $TlClO_4$, 975 mM NaCN, $(3025 - c_{Tl})$ mM $NaClO_4$ from a burette to a solution containing c_{Tl} mM $TlClO_4$ and $(4000 - c_{Tl})$ mM $NaClO_4$. From the E' values in Table 8 it is clear that no complexes are formed between thallium(I) and cyanide ions.

Table 8. Potentiometric measurements in the cyanide system.

c_{Tl} mM	c_{NaCN} mM	E mV	E_j' mV	E' mV
100	244	−0.1	0.1	0
	488	−0.3	−0.3	0
	975	−0.6	−0.6	0
50	488	−0.2	−0.3	0.1

B. Measurements of the solubility of TlA in NaA solutions

Method of evaluation of the equilibrium constants

The complex formation in the chloride, bromide, iodide and thiocyanate systems was also studied by means of solubility measurements. The solubility of TlA in NaA solutions of different concentrations was determined using a radioassay technique.

If the complex formation is written

$$m\,\mathrm{TlA}_{(s)} + x\,\mathrm{A}^- \rightarrow \mathrm{Tl}_m\,\mathrm{A}_{m+x}^{x-}$$

the system is defined by the equilibrium constants

$$k_{m,x} = [\mathrm{Tl}_m\,\mathrm{A}_{m+x}^{x-}]/[\mathrm{A}^-]^x. \tag{13}$$

For the solubility we get

$$l = \sum K_x [\mathrm{A}^-]^x, \tag{14}$$

where

$$K_x = k_{1,x} + 2k_{2,x} + 3k_{3,x} + \cdots \tag{15}$$

(*vide* Leden [17]).

As shown before, only mononuclear species are formed, and the solubility therefore can be written

$$l = \sum_{x=-1}^{N-1} k_{1,x}\,[\mathrm{A}^-]^x \quad \text{or simpler} \quad l = \sum_{x=-1}^{N-1} k_x [\mathrm{A}^-]^x, \tag{16}$$

where $k_{-1} = L$. From (2) and (13) we get

$$k_x = \beta_{x+1} \cdot L. \tag{17}$$

The equilibrium constants were evaluated by a graphical method. k_0 was obtained as ordinate when $l - L/[\mathrm{A}^-]$ *versus* $[\mathrm{A}^-]$ was extrapolated to $[\mathrm{A}^-] = 0$, k_1 from $(l - L/[\mathrm{A}^-] - k_0)/[\mathrm{A}^-]$ *vs.* $[\mathrm{A}^-]$ and so on. In the chloride and thiocyanate systems $\bar{n}$ was known from the potentiometric measurements, and consequently $[\mathrm{A}^-]$ could be calculated according to eq. (6). In the bromide and iodide systems $[\mathrm{A}^-]$ was approximately equal to c_A except at the point where $c_\mathrm{NaA} = 0$, where a correction had to be made for the undissociated ion pair TlA; thus here $[\mathrm{A}^-] = l - k_0$.

The solubility product, L, was obtained in two ways. On the one hand it could be calculated from $L = (l_0 - k_0)^2$ where l_0 is the solubility of TlA in 4 *M* $\mathrm{NaClO_4}$; on the other it was calculated, as $L = [\mathrm{Tl}^+]\,[\mathrm{A}^-]$, as a function of $[\mathrm{A}^-]$ after $[\mathrm{Tl}^+]$ had been determined by emf measurements in cell (8), the saturated solution forming solution S. The liquid junctions used in these measurements were 4 *M* $\mathrm{NaClO_4}$ and 4 *M* NaA at low and high values of c_A respectively. It was shown that E_m never altered by more than 0,3 mV when the composition of the salt bridge was changed. From the evaluated k_x values $\bar{n}$ was calculated according to

$$\bar{n} = \frac{\sum_{x=0}^{N-1} (x+1)\,k_x\,[\mathrm{A}^-]^x}{\sum_{x=-1}^{N-1} k_x\,[\mathrm{A}^-]^x} \tag{18}$$

and l according to eq. (16).

The results of the solubility measurements are collected in Tables 9, 10, 11 and 12 and in Fig. 5, 6, 7, 8 and 9.

Table 9. Solubility of TlCl in sodium chloride solutions.

$L_{sol.} = (920 \pm 30)\ 10^{-6}\ M^2$; $k_0 = (800 \pm 200)\ 10^{-6}\ M$; $k_1 = (180 \pm 40)\ 10^{-6}$; $k_2 = (20 \pm 10)\ 10^{-6}\ M^{-1}$.

c_{NaCl} mM	$l_{exp.}$ mM	$\bar{n}_{calc.}$	[Cl$^-$] mM	E_m mV	E_j mV	$L_{pot.}$ $10^4\ M^2$	$l_{calc.}$ mM
0	31.1	0.02	30.3	1,185.0		9.1	30.9
20	22.2	0.02	41.4	1,193.2		9.1	22.7
50	14.9	0.05	64.1	1,204.2		9.2	15.0
80	11.1	0.08	90.3	1,212.8		9.2	10.9
120	8.00	0.10	127	1,221.7	−0.1	9.1	8.10
160	6.23	0.14	165	1,228.5	−0.1	9.1	6.37
200	5.30	0.17	204	1,233.9	−0.1	9.1	5.33
250	4.51	0.20	254	1,239.3	−0.2	9.2	4.48
300	3.98	0.23	303	1,243.7	−0.2	9.2	3.92
400	3.23	0.29	402	1,250.8	−0.2	9.3	3.21
500	2.77	0.35	502	1,255.8	−0.3	9.5	2.78
630	2.24	0.43	631	1,261.3	−0.3	9.6	2.44
800	2.09	0.51	801	1,267.2	−0.4	9.7	2.18
1,000	2.84	0.61	1,000	1,272.1	−0.5	10.0	1.99
1,500	1.72	0.82	1,500	1,281.1	−0.7	10.4	1.81
2,000	1.81	0.98	2,000	1,287.2	−0.9	10.9	1.80
2,500	1.86	1.14	2,500	1,291.9	−1.1	11.3	1.84
3,000	1.88	1.27	3,000	1,295.3	−1.3	11.8	1.91
3,250	1.90	1.32	3,250	1,296.8	−1.4	12.0	1.97
3,500	1.99	1.38	3,500	1,298.2	−1.5	12.1	2.03
3,750	2.08	1.44	3,750	1,299.5	−1.6	12.3	2.09
4,000	2.20	1.48	4,000	1,300.8	−1.7	12.4	2.16

Table 10. Solubility of TlBr in sodium bromide solutions.

$L_{sol.} = (15.6 \pm 0.3)\ 10^{-6}\ M^2$; $k_0 = (33 \pm 5)\ 10^{-6}\ M$; $k_1 = (24 \pm 5)\ 10^{-6}$; $k_2 = (8 \pm 3)\ 10^{-6}\ M^{-1}$; $k_3 = (1.6 \pm 0.6)\ 10^{-6}\ M^{-2}$.

c_{NaBr} mM	$l_{exp.}$ 10^3 mM	$\bar{n}_{calc.}$	[Br$^-$] mM	E_m mV	E_j mV	$L_{pot.}$ 10^5	$l_{calc.}$ 10^3 mM
0	3,980	0.01	3.95	1,237.2		1.56	3,983
10	1,370	0.02	11.4	1,264.7		1.54	1,403
40	379	0.08	40.4	1,297.7		1.52	420
100	187	0.20	100	1,320.2	−0.1	1.56	190
200	113	0.37	200	1,338.5	−0.1	1.53	116
300	81.0	0.54	300	—	—	—	93.4
500	89.9	0.81	500	—	—	—	78.8
700	82.4	1.05	700	1,369.3	−0.4	1.60	76.5
1,000	86.5	1.34	1,000	1,377.4	−0.6	1.65	82.2
1,500	102	1.74	1,500	1,387.9	−0.9	1.62	103
2,000	126	2.05	2,000	1,394.5	−1.1	1.66	134
2,500	170	2.30	2,500	1,399.2	−1.4	1.71	174
3,000	227	2.49	3,000	1,403.2	−1.7	1.74	225
3,500	294	2.66	3,500	1,406.4	−2.0	1.77	286
4,000	360	2.78	4,000	1,410.0	−2.2	1.74	361

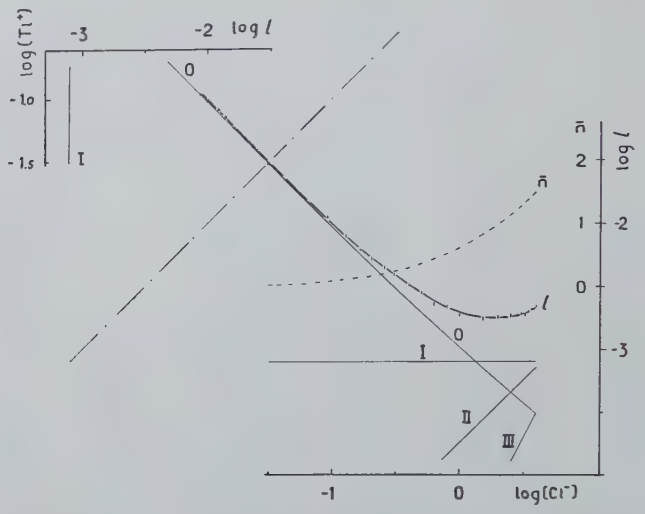

Fig. 5. log l and $\bar{n}$ *vs.* log [Cl$^-$] (lower right part of the figure). | | | solubility of TlCl in NaCl solutions. The concentrations of the complexes at a given value of [Cl$^-$] can be read from the straight lines. The roman figures denote the number of ligands attached to the thallium ion. The solubility and $\bar{n}$ curves are calculated with the evaluated k_x values (see Table 9). × × × solubility of TlCl in $TlClO_4$ solutions (upper left part of the figure). The solubility here is the sum of [TlCl] (the line marked I) and [Cl$^-$] (the line marked 0).

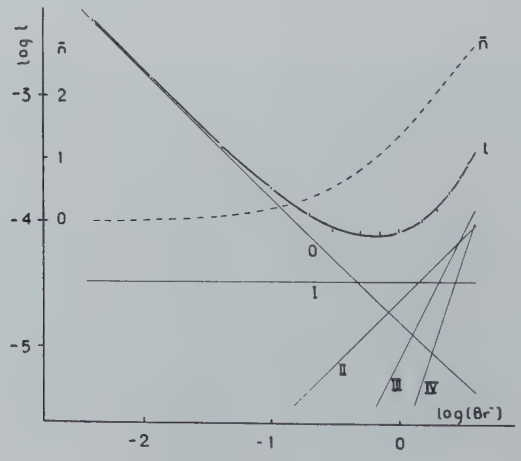

Fig. 6. log l and $\bar{n}$ *vs.* log [Br$^-$]. For explanation see text of Fig. 5.

Experimental

TlCl, TlBr, TlI, and TlSCN were precipitated from hot thallium(I) nitrate solutions containing some $^{204}Tl^+$ (β-emitter, $t_{\frac{1}{2}} = 2.7$ years) ions. After aging the precipitates were thoroughly washed and transferred to glass tubes containing solutions of the compositions c_{NaA} mM NaA; $(4000 - c_{NaA})$ mM $NaClO_4$, where A stands for the halide

Table 11. Solubility of TlI in sodium iodide solutions.

$L_{sol.} = (0.189 \pm 0.005)\ 10^{-6}\ M^2$; $k_0 = (1.0 \pm 0.1)\ 10^{-6}\ M$; $k_1 = (1.5 \pm 0.4)\ 10^{-6}$; $k_2 = (2.3 \pm 0{,}5)\ 10^{-6}\ M^{-1}$; $k_3 = (1.0 \pm 0.2)\ 10^{-6}\ M^{-2}$.

c_{NaI} mM	$l_{exp.}$ 10^3 mM	$\bar{n}_{calc.}$	[I$^-$] mM	E mV	E_j mV	$L_{pot.}$ $10^7\ M^2$	$l_{calc.}$ 10^3 mM
0	436		0.43	1,294.0		1.88	436
1	159	0.01	1.16	1,320.2		1.81	163
2	88.5	0.01	2.09	1,334.2		1.90	91.4
5	38.1	0.03	5.04	~1,355		(2.0)	38.6
10	18.5	0.06	10.0	~1,370		(2.3)	19.8
20	10.1	0.10	20.0	~1,391		(2.0)	10.4
50	4.76	0.25	50.0	~1,415		(2.0)	4.85
100	2.95	0.45	100	1,434.2	−0.1	1.84	3.06
200	2.25	0.82	200	1,451.7	−0.1	1.87	2.34
250	2.16	1.00	250	1,456.7	−0.2	1.91	2.30
500	2.97	1.68	500	1,474.4	−0.3	1.92	2.85
800	4.54	2.24	800	1,486.9	−0.4	1.87	4.44
1,200	8.10	2.69	1,200	1,496.5	−0.5	1.92	8.07
1,500	12.5	2.92	1,500	1,502.7	−0.8	1.87	12.0
2,000	20.8	3.14	2,000	1,509.8	−1.0	1.88	21.6
2,500	33.7	3.28	2,500	1,515.1	−1.2	1.89	35.3
3,000	52.5	3.40	3,000	1,520.2	−1.4	1.85	54.1
3,500	79.2	3.48	3,500	1,524.4	−1.6	1.82	78.1
4,000	111	3.54	4,000	1,527.7	−1.8	1.82	110

Table 12. Solubility of TlSCN in sodium thiocyanate solutions.

$L_{sol.} = (690 \pm 20)\ 10^{-6}\ M^2$; $k_0 = (1000 \pm 200)\ 10^{-6}\ M$; $k_1 = (700 \pm 100)\ 10^{-6}$; $k_2 = (250 \pm 50)\ 10^{-6}\ M^{-1}$; $k_3 = (107 \pm 30)\ 10^{-6}\ M^{-2}$.

c_{NaSCN} mM	$l_{exp.}$ mM	$\bar{n}_{calc.}$	[SCN$^-$] mM	E mV	E_j mV	$L_{pot.}$ $10^4\ M^2$	$l_{calc.}$ mM
0	27.4	0.04	26.4	1,188.0	—	7.06	27.9
0	27.1	0.04	26.1	1,187.8	—	7.02	28.2
30	16.2	0.06	45.2	1,202.0	—	7.03	16.7
70	9.67	0.11	78.7	1,216.0	—	7.09	10.1
100	7.45	0.15	106	1,223.6	—	7.11	7.77
300	3.36	0.41	302	1,248.8	—	7.60	3.58
500	2.88	0.69	501	1,262.9	—	7.26	2.85
700	2.68	0.94	700	1,271.1	—	7.39	2.66
900	2.72	1.17	900	1,277.7	0.1	7.38	2.70
1,100	2.84	1.40	1,100	1,282.7	0.1	7.44	2.87
1,300	3.27	1.61	1,300	1,286.9	0.1	7.46	3.11
1,500	3.45	1.81	1,497	1,290.4	0.2	7.52	3.44
1,700	3.83	1.98	1,696	1,293.6	0.2	7.52	3.86
2,000	4.75	2.21	1,994	1,297.5	0.2	7.60	4.60
2,300	5.65	2.41	2,291	1,301.1	0.2	7.58	5.50
2,700	6.85	2.64	2,687	1,305.1	0.3	7.63	7.03
3,000	8.30	2.77	2,986	1,307.8	0.3	7.66	8.41
3,300	9.97	2.88	3,280	1,310.1	0.3	7.69	9.98
3,700	12.0	3.02	3,673	1,313.2	0.4	7.65	12.45
4,000	14.1	3.10	3,966	1,315.0	0.4	7.70	14.58

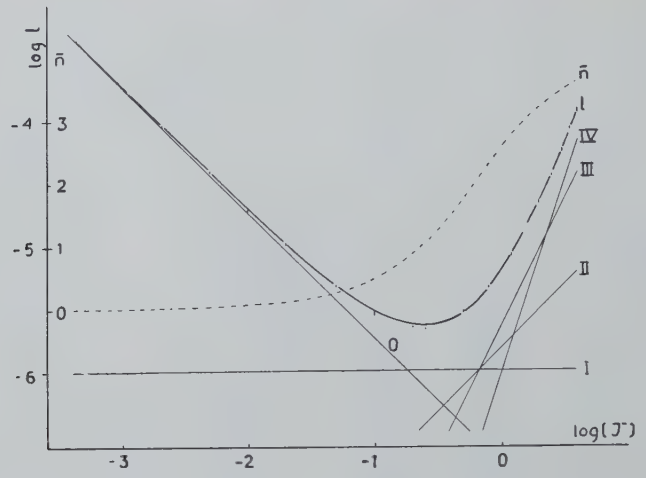

Fig. 7. log l and $\bar{n}$ *vs.* log [I^-]. For explanation see text of Fig. 5.

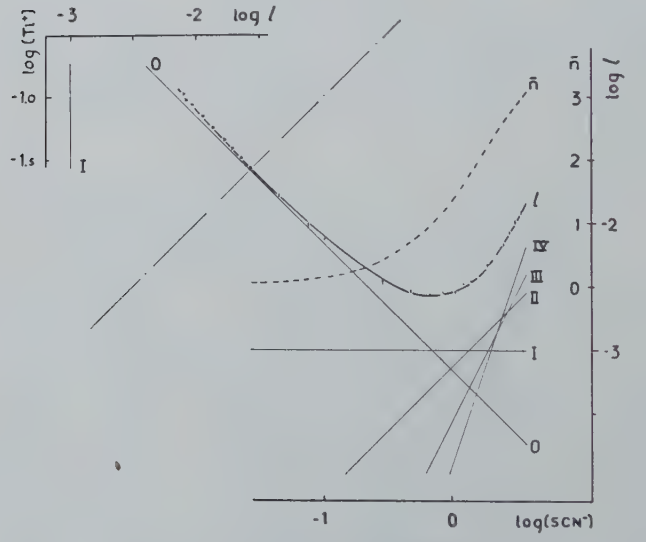

Fig. 8. log l and $\bar{n}$ *vs.* log [SCN^-]. For explanation see text of Fig. 5.

corresponding to the halide in the precipitate. Before the tubes were rubberstoppered and paraffined, nitrogen was passed through those solutions which belonged to the iodide system to prevent oxidation of the iodide ions by air. The tubes were rotated three to six days in a waterthermostat at 25°C. The saturated solutions were then pressed through membrane filters (finest) in a thermostatically controlled Thiessen apparatus. The first 5 ml of the filtrate were discarded to avoid errors owing to adsorption in the filter apparatus. The filtrate was then divided into 10 ml portions in which the radioactivity was measured with an alcoholquenched liquid sampling counter

tube. In the same counter tube the radioactivity of 10 ml portions of the initial $^{204}Tl^+$-containing thallium nitrate solutions was measured after sodium perchlorate had been added to the ionic strength 4 M. c_{Tl} in the saturated solutions could then be calculated. Consideration was taken to the fact that the β-absorption was not the same in 4 M $NaClO_4$ as in those saturated solutions in which a great part of the perchlorate ions had been exchanged by halide ions. X-ray photographs taken with a Guinier camera of the precipitates remaining in the tubes at equilibrium showed, that no other solid phases than those with the composition TlA existed in the concentration ranges investigated.

C. Measurements of the solubility of TlA in thallium perchlorate solutions

The solubilities of TlCl and TlSCN were determined in solutions with the compositions c_{TlClO_4} mM $TlClO_4$, $(4000 - c_{TlClO_4})$ mM $NaClO_4$ in the range $0 \leqq c_{TlClO_4} \leqq 100$ mM where the upper limit was set by the low solubility of $TlClO_4$. In the thiocyanate system the solubility was determined by Volhard titration and in the chloride system by titration with KIO_3 in 5 M HCl at which Tl(I) was oxidized to Tl(III). $[Tl^+]$ was determined in the saturated solutions by emf measurements in cell (8).

If the complex formation is written

$$n\,TlA_{(s)} + y\,Tl^+ \rightleftharpoons Tl_{n+y}\,A_n^{y+}$$

the system is defined by the equilibrium constants

$$e_{n,y} = [Tl_{n+y}\,A_n^{y+}] / [Tl^+]^y. \tag{19}$$

If only species with $n = 1$ are formed we get

$$l = \frac{L}{[Tl^+]} + \sum_{y=0} e_y\,[Tl^+]^y. \tag{20}$$

From (13) and (19) it is found that $e_0 = k_0$.

When l was plotted against $1/[Tl^+]$ straight lines (with slope L and origo ordinate e_0) were obtained in both the chloride and the thiocyanate systems. This indicates that the only complex existing in the concentration range investigated is TlA. It

Table 13. Solubility of TlCl in thallium perchlorate solutions.
$L = (915 \pm 20)\ 10^{-6}\ M^2$; $e_0 = (700 \pm 200)\ 10^{-6}\ M$.

c_{TlClO_4} mM	l mM	E_m mV	$[Tl^+]$ mM
0	30.8	1,185.1	30.0
0	31.2	1,185.1	30.0
14.8	24.1	1,179.0	38.0
44.4	16.1	1,167.6	59.3
59.2	13.5	1,162.6	72.1
69.1	12.3	1,159.8	80.3
79.0	11.0	1,157.2	88.9
88.8	10.1	1,154.7	97.9
98.7	8.7	1,152.5	106.5

was found that in the chloride system $e_0 = (700 \pm 200)\,10^{-6}$ M and $L = (915 \pm 20)\,10^{-6}$ M^2 and in the thiocyanate system $e_0 = (1000 \pm 200)\,10^{-6}$ M and $L = (720 \pm 20)\,10^{-6}$ M^2, which are in good agreement with the results previously obtained. The results of the measurements are collected in Tables 13 and 14.

D. Discussion

The equilibrium constants determined in this paper are given as β constants in Table 15.

Owing to the low tendency to complex formation shown by the thallium ion, the measurements in both the potentiometric and the solubility investigations were carried out at halide concentrations up to 4 M. To get an idea of how much the activity factors varied, when the perchlorate ions were exchanged by halide ions, the solubility product, L, was determined potentiometrically as a function of $[A^-]$. From Fig. 9 it may be seen that L is constant in the iodide system and increases about 10 % in the bromide and thiocyanate systems and about 30 % in the chloride system. Accordingly the iodide constants may be regarded as true. In the thiocyanate system both the solubility measurements and the potentiometric measurements could be described with the same constants in spite of the fact that the activity factors operate in different ways by these two methods. This fact may be used to illustrate that the variation of the activity factors is of little significance when the perchlorate ions are exchanged by thiocyanate ions. Consequently the bromide constants may be regarded as fairly true too, as the variation of L in the bromide system is not greater than that in the thiocyanate system. In the chloride system, on the other hand, the potentiometric measurements could be described by assuming the presence of the species Tl^+, $TlCl$ and $TlCl_2^-$, while to describe the solubility measurements it was necessary to assume the presence of $TlCl_3^{2-}$ as well. This disagreement must be ascribed to a variation of the activity factors, and it is accordingly difficult to say which of the two sets of constants is the more correct one. Scott and Hu (2), however, by spectrophotometric measurements proved the existence of species with more than two ligands at potassium chloride concentrations > 3 M, and it is therefore possible

Table 14. Solubility of TlSCN in thallium perchlorate solutions.

$L = (720 \pm 20)\ 10^{-6}\ M^2$; $e_0 = (1000 \pm 200)\ 10^{-6}\ M$.

c_{TlClO_4} mM	l mM	E_m mV	$[Tl^+]$ mM
0	27.7	1,187.9	26.9
0	27.7	1,187.8	27.0
10.0	23.1	1,183.1	32.4
19.9	19.5	1,178.4	38.9
29.9	16.8	1,174.2	45.8
44.8	13.4	1,168.4	57.4
59.7	11.4	1,163.2	70.2
69.7	10.4	1,160.3	78.7
79.6	9.4	1,157.6	87.3
89.6	8.5	1,155.1	96.4
99.5	8.0	1,152.7	105.8

Table 15. Constants determined in this work at the ionic strength 4 M and at 25°C. β_n defined by eq. (2). The evaluated k_x constants (eq. 13) recalculated to β_n constants according to eq. (17). $b_1 = \beta_1$; $b_2 = \beta_2/\beta_1$.

Ligand	Method of measurement	$\beta_1 = b_1$ M^{-1}	β_2 M^{-2}	β_3 M^{-3}	β_4 M^{-4}	L $10^6\ M^2$	b_2 M^{-1}	$\frac{b_1}{b_2}$
F^-	pot.	0 [1]						
Cl^-	pot.	1.0 ± 0.1	0.16 ± 0.05			910 ± 20 [2]	0.16	6.3
						1240 ± 20 [3]		
	sol. in NaCl	0.8 ± 0.2	0.16 ± 0.05	0.02 ± 0.01		920 ± 30	0.20	4.0
	sol. in $TlClO_4$	0.8 ± 0.2				915 ± 20		
Br^-	pot.					15.5 ± 0.3 [2]		
						17.5 ± 0.3 [3]		
	sol. in NaBr	2.1 ± 0.4	1.4 ± 0.3	0.5 ± 0.2	0.09 ± 0.04	15.6 ± 0.3	0.67	3.1
I^-	pot.					0.187 ± 0.005 [2]		
						0.187 ± 0.005 [3]		
	sol. in NaI	5.3 ± 0.5	8 ± 2	12 ± 3	5 ± 1	0.189 ± 0.005	1.5	3.5
SCN^-	pot.	1.4 ± 0.2	1.0 ± 0.2	0.35 ± 0.05	0.12 ± 0.04	710 ± 10 [2]	0.72	1.9
						770 ± 10 [3]		
	sol. in NaSCN	1.4 ± 0.3	1.0 ± 0.2	0.33 ± 0.06	0.14 ± 0.05	690 ± 20		
	sol. in $TlClO_4$	1.4 ± 0.3				720 ± 20		
CN^-	pot.	0						

[1] Ionic strength 1 M.
[2] For low c_A values (*vide* Fig. 9).
[3] For $c_A = 4\ M$.

that in the chloride system the constants evaluated from the solubility measurements are more reliable than those evaluated from the potentiometric measurements.

In Figs. 5–8 log l is plotted *vs.* log $[A^-]$. The full-drawn lines are calculated from the k_x values in Tables 9–12. In the same diagrams the concentrations of the different complexes at a given value of $[A^-]$ can be read from the straight lines marked 0–IV, where the roman figures denote the number of ligands attached to the thallium ion. The percentile distribution of the complexes in unsaturated solutions is the same as in the saturated ones as only mononuclear species are present. It is obvious from Figs. 5–8 that a certain complex never dominates completely. For example, in the iodide system the ion pair TlI contributes about 43 % of the solubility at $[I^-] = 250$ mM, where the solubility is at a minimum; while $[Tl^+]$ amounts to 33 %. When the iodide concentration is 650 mM each of the species TlI, TlI_2^-, and TlI_3^{2-} accounts for about 28 % of the soubility and when $[I^-]$ has reached its highest value, 4 M, the fourth complex TlI_4^{3-} accounts for 60 % of the soubility and TlI_3^{2-} for 33 %. It is clear that this fact makes the evaluation of the equilibrium constants more difficult, than if each of the complexes had dominated over a certain range. Under these conditions the possibility exists of taking one constant too high at the expense of the following and nevertheless be able to describe the measurements with the evaluated constants. As a consequence the limits of error are rather wide, especially for the complexes with three and four ligands. The $\bar{n}$ curves in Figs. 5–8 also rise without forming the "plateaus", which would have been found if some of the complexes had dominated over certain ranges. It is obvious that the complex formation is uncompleted at $[A^-] = 4\ M$, and therefore it is not possible to say which is the maximum coordination number of thallium.

In Table 15 the stability constant $b_2 = \beta_2/\beta_1$ and the quotient b_1/b_2 also are

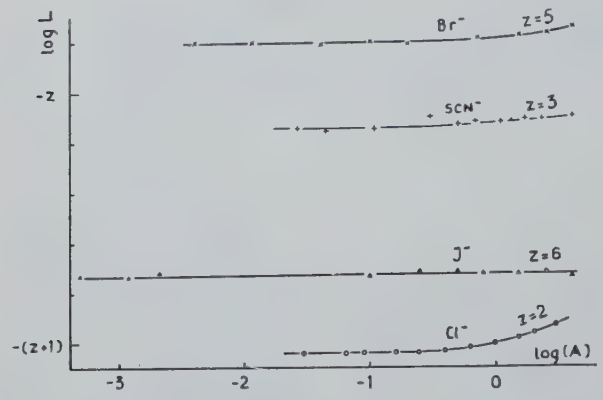

Fig. 9. log L *vs.* log [A].

calculated. b_1, b_2 and (ignoring the chloride system where the activity changes are uncertain) b_1/b_2 rise in the order $F < Cl < Br < I$ indicating that Tl^+ belongs to the group of metal ions, Cu^+, Ag^+, Cd^{2+}, Hg^{2+} and probably Tl^{3+} (*vide* for example Carleson and Irving [18]), in which the strength of the halide complexes increases with increasing atomic weight of the halide ion.

The thallium(I) does not form cyanide complexes, in contrast to the above-mentioned metals, and resembles in this respect the alkali metals.

Summary

The complex formation in the thallium halide, thiocyanate and cyanide systems has been studied at 25°C. The ionic strength was kept constant at 4 M with $NaClO_4$ as an additional salt. Only mononuclear complexes were found to exist in the ranges investigated. The equilibrium constants were determined by two methods.

1. Potentiometric measurements of $[Tl^+]$ with a thallium amalgam electrode in solutions with the same total thallium content but different halide concentrations. The β values [defined in eq. (2)] are found in Table 15.

2 *a*. Determination of the solubility of TlCl, TlBr, TlI and TlSCN in NaA solutions ($0 \leq c_{NaA} \leq 4$ M) with a radioassay technique. The evaluated k_x values [*vide* eq. (13)] can be seen in Tables 9–12 and, recalculated to β constants, in Table 15, where also the solubility products L calculated from the solubility of TlA in 4 M $NaClO_4$ are found.

b. Determination of the solubility of TlCl and TlSCN in $TlClO_4$ solutions in the range $0 \leq c_{TlClO_4} < 100$ mM. The only complex of the type $Tl_mA^{(m-1)+}$ which existed in this concentration range was TlA. The evaluated L values and e_0 constants, the latter recalculated to β constants, are given in Table 15.

No complex formation was found to take place in the fluoride and cyanide systems. It was established that the stability of the thallium(I) halide complexes increases in the order $F < Cl < Br < I$. The distribution of the complexes formed can be seen in Figs. 5–8.

To get measure on the variation of the activity factors when the perchlorate ions were exchanged by halide ions, L was determined potentiometrically as a function of $[A^-]$ (Fig. 9). It was found that the inconsistency of the activity factors was serious only in the chloride system.

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Department of Inorganic Chemistry, Chalmers Institute of Technology, Göteborg, Sweden.

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Effect of glutamine on the synthesis of mucopolysaccharides in the nucleus pulposus *in vitro*

By Lennart Rodén

With 1 figure in the text

The nucleus pulposus contains an unusually large amount of polysaccharides, up to 60 per cent of the dry weight in some animal species (1). Chondroitin sulphuric acid (2) and a keratosulphate-like fraction have been isolated (3–5). In addition, small amounts of fucose and mannose are present, probably as constituents of glycoproteins (5).

It has recently been shown that glutamine exerts a stimulating effect on the synthesis of chondroitin sulphuric acid in slices of cartilage (6, 7), probably acting as the nitrogen donor for hexosamine synthesis. In order to extend the investigation of the glutamine effect to other tissues and other mucopolysaccharides, the carbohydrate formation in the nucleus pulposus has been studied *in vitro*.

Experimental

Material

Nuclei pulposi from pigs were removed from the lower lumbar and sacral intervertebral discs within 1 hour of slaughter and cooled on ice. About 5–6 g (from 50–60 pigs) were used in each experiment.

Methods

In vitro method for isotope incorporation

2–3 g of nucleus was suspended in 20 ml of Krebs–Ringer bicarbonate solution (8) and shaken vigorously to divide the tissue as finely as possible. The samples were rocked in a water bath at 37°C for varying periods of time in the presence of S^{35}-labelled sulphate or uniformly C^{14}-labelled glucose, and aerated concurrently with an oxygen-carbon-dioxide mixture (93.5 and 6.5 per cent, respectively).

Preparation of the polysaccharides[1]

A mixture of polysaccharides was prepared essentially as described by Gardell and Rastgeldi (3). The method consists of digestion with proteolytic enzymes (glycerol

[1] Carried out in the S^{35} experiments only.

extracts of pig pancreas and pig intestinal mucosa) and extraction of the ethanol-precipitable material with 90 per cent phenol. The phenol-insoluble fraction contains the polysaccharide material. In the present work, the ethanol precipitate was dialyzed against running distilled water for 35 hours at room temperature, and freeze-dried prior to extraction with phenol. The radioactivity was measured at infinite thickness, and the material was subsequently dissolved in water and reprecipitated with ethanol to constant specific activity. The absence of free S^{35}-labelled sulphate was checked by paper electrophoresis (9).

Fractionation of the polysaccharide mixture

The material was fractionated with the technique introduced by Scott (10) and Stacey (11), using quaternary ammonium compounds. The method was essentially that of Borelius, Gardell and Scott (12).[1]

The keratosulphate-like fraction was isolated by dissolving the polysaccharide mixture (about 160 mg) in a few ml of water and precipitating the chondroitin sulphuric acid with an excess of 1 per cent cetyl pyridinium chloride. The precipitate was removed by centrifugation at 3000 *g* for 10 minutes, and the supernatant was finally filtered through a glass filter.

Chondroitin sulphuric acid was obtained in a pure form by the following procedure. The crude polysaccharide mixture (about 160 mg) was dissolved in 2.5 ml of water and added to the top of a 1×20 cm cellulose column, previously washed with water and 1 per cent cetyl pyridinium chloride. The column was eluted with 1 per cent cetyl pyridinium chloride, half-hour fractions being collected (4–6 ml). After fraction 45, the eluent was changed to 1.5 *N* KCl. The effluent was analyzed for uronic acid and hexoses with the methods of Dische (13, 14).

Due to overloading of the column, the "keratosulphate", which emerged in the first fractions, was contaminated with chondroitin sulphuric acid. This could be completely removed by the addition of more 1 per cent cetyl pyridinium chloride and centrifugation.

The amounts of "keratosulphate" obtained did not permit an exhaustive characterization of the polysaccharide. The method of preparation has, however, been shown by Borelius, Gardell and Scott (12) to yield a keratosulphate-like material of high purity. Furthermore, the analyses (15, 16) showed the presence of glucosamine and galactose in approximately equimolar quantities. Of the total hexosamine, about 90 per cent was glucosamine.

In the first fractions eluted with KCl, a precipitate containing chondroitin sulphuric acid had formed; it was dissolved by addition of a small amount of 1.5 *N* KCl. The solution was diluted with several volumes of 1 per cent cetyl pyridinium chloride and the resultant precipitate spun down at 3000 *g*. It was dissolved in 3 *M* $MgCl_2$ and reprecipitated with 1 per cent cetyl pyridinium chloride. The precipitate was once more spun down and dissolved in a small amount of $MgCl_2$, precipitated with ethanol, washed and dried with ethanol and ether. The uronic acid content of two samples of chondroitin sulphuric acid prepared in this way (carbazole method (13)) was 126 and 88 per cent, respectively, of the value found for a sample of chondroitin sulphuric acid prepared from calf costal cartilage according to the method of Boström and Månsson (17).

[1] I wish to thank these authors for permission to use their method prior to its publication.

Determination of radioactivity

Aliquots of solutions of the keratosulphate-like fraction and chondroitin sulphuric acid containing approximately 25 μg of galactose (cysteine method (14)) and 100 μg of chondroitin sulphuric acid, respectively, were plated on 3.1 cm^2 aluminium planchets, and the radioactivity measured in a Tracerlab SC-16 windowless flow counter.

Incorporation of C^{14}-glucose

The uptake of uniformly labelled C^{14}-glucose in the nucleus pulposus was also studied. After incubation as described above, the samples were rapidly frozen and kept overnight at −15°C. They were subsequently dialyzed against running distilled water for 24 hours, and the polysaccharide-containing material precipitated by the addition of several volumes of ethanol. The precipitate was collected by centrifugation, dried with ethanol and ether, and divided into two similar portions for the isolation of neutral monosaccharides and hexosamines, respectively.

Neutral monosaccharides.—These were isolated according to a modification (18) of Gardell's chromatographic method (16) on a 1 × 20 cm starch column, after hydrolysis with 1 *N* H_2SO_4 at 100°C for 12 hours. The modifications were as follows:

(1) Removal from the hydrolysate of ions and incompletely hydrolyzed oligosaccharide material on a column composed of (*a*) Zeo-Karb 215, 4.5 per cent cross linkage, 2 × 5 cm in H^+ form, (*b*) Dowex 2, 8 per cent cross linkage, 2 × 9 cm in acetate form, and (*c*) decolorizing carbon (Hopkin & Williams) 2 × 0.5 cm.

(2) Changing the proportions of the chromatography solvent butanol–propanol–water to 2 : 3 : 1 instead of 4 : 1 : 1. In the chromatographic separation, 2-hour fractions (1–2 ml) were collected; 0.5 ml was taken for the colorimetric analysis (19) and 0.5 ml was plated on aluminium planchets for determination of the radioactivity in the flow counter.

Hexosamines.—These were separated by Gardell's method (15) on 2 × 40 cm columns of Dowex 50, after hydrolysis with 6 *N* HCl at 100°C for 8 hours. 2-hour fractions (10-15 ml) were collected, and 0.5 ml aliquots were taken for colorimetric analysis. The radioactivity of 0.5 ml aliquots was measured, using lead planchets, in a Tracerlab Superscaler with an end-window tube.

Results

S^{35} incorporation

S^{35}-sulphate was incorporated into the keratosulphate-like fraction as well as into the chondroitin sulphuric acid. In the presence of glutamine, the incorporation was greatly increased in both compounds (Table 1).

C^{14} incorporation

Neutral monosaccharides.—Starch chromatography resolved the hydrolysate of nucleus pulposus incubated with C^{14}-glucose into several sugar-containing components. Three of these emerged in the same effluent volumes as mannose, glucose and galactose, respectively. They closely corresponded to peaks of radioactivity, the largest of which emerged at the place of glucose (Fig. 1).

A comparison of the control sample with that incubated with glutamine showed an increase in specific activity of the galactose and mannose in the latter (Table 2).

Table 1. Influence of glutamine on incorporation of S^{35}-sulphate in nucleus pulposus polysaccharides.

Each of two flasks contained 2.7 g nucleus pulposus in 20 ml Krebs–Ringer bicarbonate buffer. To one flask 4.0 mg glutamine and 25 mg glucose were added. Incubation at 37°C for 3.5 hours. After addition of 250 μC S^{35}-labelled sulphate in 5 ml buffer, incubation was continued for 12.5 hours.

	S^{35} activity* c.p.m.	
	Chondroitin sulphuric acid	"Kerato-sulphate"
Control	699	1538
Glutamine + glucose added .	9150	6799

* Radioactivity of 503 μg chondroitin sulphuric acid and 467 μg "keratosulphate" (corresponding to 1 μM of the disaccharide units as the sodium salts).

Table 2. Influence of glutamine on incorporation of C^{14}-glucose in neutral monosaccharides of nucleus pulposus.

Each of two flasks contained 2.2 g nucleus pulposus in 20 ml Krebs–Ringer bicarbonate buffer. To one flask 3.9 mg glutamine was added. Incubation for 2 hours at 37°C. After addition of 20 μC C^{14}-glucose (specific activity 115 μC/mg) in 5 ml buffer, incubation was continued for 5.3 hours.

	Relative specific activity $\frac{\text{c.p.m.}}{\varepsilon_{370} \times 10^3}$	
	Galactose	Mannose
Control	0.42	3.6
Glutamine added	1.03	5.2

Table 3. Influence of glutamine on incorporation of C^{14}-glucose in hexosamines of nucleus pulposus.

For experimental details, see Table 2.

	Relative specific activity $\frac{\text{c.p.m.}}{\varepsilon_{535} \times 10^3}$	
	Glucosamine	Galactosamine
Control	0.36	0.08
Glutamine added	0.85	0.24

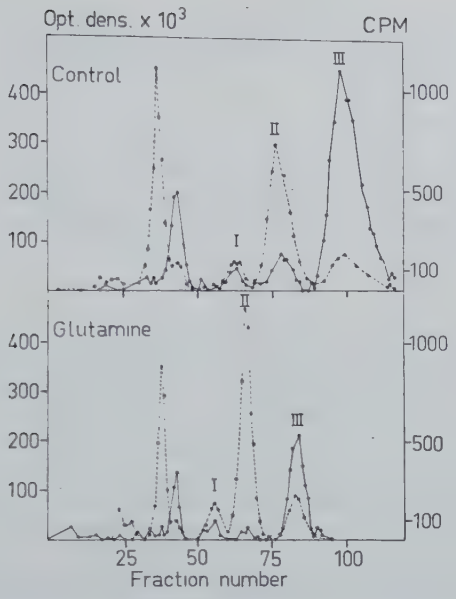

Fig. 1. Chromatographic separation of neutral monosaccharides from nucleus pulposus after incubation with C^{14}-glucose. • ——— • Optical density at 370 mμ, • – – – • Radioactivity. Peaks I–III correspond to mannose, glucose and galactose, respectively. For experimental details, see Table 2.

Hexosamines.—The specific activity of the hexosamines from normal and glutamine-stimulated nucleus is recorded in Table 3. In the control sample, the specific activity of the glucosamine was approximately 5 times higher than that of galactosamine. Increases in activity of both glucosamine and galactosamine were observed in the glutamine-stimulated sample, amounting to 2.4 and 3.0 times the control values, respectively.

Discussion

The addition of glutamine to the incubation medium resulted in a higher uptake of the isotopic precursors (S^{35}-sulphate and C^{14}-glucose) in the components of both polysaccharides. Although the mechanism of stimulation has not been established, it seems highly probable that glutamine plays the same role as in the synthesis of glucosamine by microorganisms, viz., that of the nitrogen donor. The increase in specific activity of the mannose, which is probably a constituent of glycoproteins in the nucleus pulposus, might also result from the participation of glutamine in the hexosamine synthesis.

A major obstacle in the study of intermediate reactions in the synthesis of mucopolysaccharides is the lack of suitable *in vitro* systems. Animal tissues in general have low activity in synthesizing mucopolysaccharides as compared to bacterial production of hyaluronic acid. The use of tracer techniques makes it possible, however, to study even the minute synthesis occurring in different mesenchymal tissues. The presence of fairly large amounts of a keratosulphate-like compound in the nucleus

pulposus makes this tissue suited for a study of the synthesis of this component. In the cornea, keratosulphate is present in far smaller amounts (20), which renders metabolic studies more difficult.

In view of the recent demonstration of hyaluronic acid synthesis in a homogenate of Rous chicken sarcoma (21), it does not seem improbable that the nucleus pulposus could constitute the basis for a study of intermediate reactions in the formation of keratosulphate and chondroitin sulphuric acid in a cell-free system. The ease with which the nucleus can be homogenized as compared to e.g. cartilage, is a great advantage.

Summary

The formation of carbohydrates in the pig nucleus pulposus *in vitro* has been studied, with special attention focused on the effect of glutamine on the biosynthesis of the mucopolysaccharides, chondroitin sulphuric acid and a keratosulphate-like component. The presence of glutamine in the incubation medium is found to result in higher incorporation of S^{35}-sulphate into both polysaccharides.

Glutamine is also found to increase the utilization of C^{14}-glucose for the synthesis of mannose, galactose, glucosamine and galactosamine, which were isolated from a hydrolysate of nucleus pulposus and identified chromatographically.

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Chemistry Department II, Karolinska Institutet, Stockholm, Sweden.

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ARKIV FÖR KEMI Band 10 nr 27

Mitgeteilt am 10. Oktober 1956 durch The Svedberg und Arne Fredga

Über Meniskuskurven benetzender Flüssigkeiten

Von Carl Drucker

Der Verlauf der Meridionalkurve eines Meniskus, welchen eine benetzende Flüsigkeit in einem kreiszylindrischen Rohre bildet, ist bisher noch nicht experimentell bestimmt worden.

Es wurden deshalb einige in Glasrohren gebildete Menisken photographisch aufgenommen. Nachdem Vorversuche die Erwartung bestätigt hatten, dass der Unterschied der Brechungsindices von Glas und Flüssigkeit eine wesentliche Rolle spielt und brauchbare Ergebnisse nur bei Übereinstimmung beider Grössen erhalten werden können, haben nach einem Vorschlage von Prof. Stig Claesson die Herren Sixten Abrahamsson, Georg Andersson und Anders Mård Flüssigkeitsgemische untersucht, deren Brechungsindices genau auf den des Glases eingestellt waren, und ihre Ergebnisse für die hier folgende Bearbeitung freundlichst zur Verfügung gestellt.

Versuchsanordnung und Resultate

Das unten geschlossene Glasrohr von bekanntem innerem Radius r Millimeter wurde in einen Trog mit planparallelen Glaswänden eingetaucht, der mit demselben Gemisch gefüllt war, sodann der Meniskus mit bekannter — etwa vierfacher — Vergrösserung photographiert und das Negativ im Comparator durchgemessen. Dessen Genauigkeit konnte nicht voll ausgenutzt werden, weil in einigen Fällen, besonders nahe am Rande, die Grenze nicht ausreichend scharf war und sich an diesen Stellen offenbar fehlerhafte Ordinatenwerte ergaben. Die Ursache dürfte in noch nicht aufgeklärten, wahrscheinlich durch optische Inhomogenität des Glases verursachten Reflexions- oder Refraktionseffekten zu suchen sein.

Ferner wurden bestimmt das Mischungsverhältnis der jeweils untersuchten binären Flüssigkeit, ihr Brechungsindex n, sowie ihr spezifisches Gewicht ϱ, und ihre Kapillaritätskonstante a^2 bei 25°, diese nach der Steighöhenmethode. Das Kapillarimeter hatte ein enges Rohr von 0,14 mm Radius und ein weites von 5 mm, die Höhen wurden bei Versuch 1 und 2 auf 0,01 mm genau abgelesen, bei den anderen nur auf 0,1 mm, was ausreichend war. Die Meniskuskorrektion des engen Rohres wurde zu 0,05 mm angenommen, die des weiten nach der früher [1] angegebenen Formel berechnet.

Bei Versuch 1 und 2 bestand das Gemisch aus Chlorbenzol (b Volumenprozent) und Cyklohexan, bei den vier anderen aus Anilin (b Volumenprozent) und Äthylalkohol.

In den folgenden Tabellen 2–7 finden sich die Ordinaten über der Basislinie y und die Abszissen x, von der Rohraxe an gezählt, beide in mm, wobei y den Mittelwert der rechts und links vom Zentrum gefundenen Zahlen darstellt.

Tabelle 1.

Versuch	n	ϱ	a^2	b	r
1	1,4732	0,9360	5,900	0,515	12,52
2	1,4739	0,9367	5,901	0,505	10,01
3	1,5122	0,9471	$7,57_1$	0,830	$9,72_5$
4	1,4784	0,9135	$6,35_4$	0,479	8,59
5	1.4784	0,9135	$6,35_4$	0,479	6,29
6	1,4774	0,9086	$6,32_7$	0,461	$3,84_5$

Tabelle 2. Versuch 1. Chlorbenzol–Cyklohexan, $r = 12{,}52$.

Nr.	x	y	Nr.	x	y
1	—	—	14	11,48	$1,04_2$
2	12,50	$2,37_4$	15	34	0,954
3	46	170	16	10,82	709
4	44	097	17	31	536
5	37	1,946	18	9,80	426 (?)
6	31	822	19	27	309
7	25	725	20	02	271
8	18	626	21	7,73	130
9	12	535	22	6,44	072
10	11,99	415	23	5,16	035
11	86	301	24	3,87	014
12	73	214	25	2,58	004
13	60	121	26	1,29	—

Tabelle 3. Versuch 2. Chlorbenzol–Cyklohexan, $r = 10{,}01$.

Nr.	x	y	Nr.	x	y
1	—	—	14	$8,07_6$	0,605
2	$9,97_0$	$2,17_3$	15	$7,82_5$	532
3	90_6	020	16	571	450
4	845	1,904	17	319	399
5	78_1	793	18	6,816	301
6	70_5	704	19	310	226
7	590	553	20	$5,80_5$	164
8	464	419	21	301	123
9	340	300	22	4,796	0,090
10	086	107	23	290	065
11	8,835	0,947	24	$3,78_6$	046
12	582	815	25	$2,52_4$	018
13	330	704	26	$1,26_2$	005

Tabelle 4. Versuch 3. Anilin–Äthylalkohol, $r = 9{,}72_5$, $A = 1{,}42$.

Nr.	x	y	$y_{ber.}$	Nr.	x	y	$y_{ber.}$
1	—	—		26	7,017	0,489	0,481
2	9,707	2,629		27	6,772	429	429
3	696	512		28	515	376	368
4	672	407	$2{,}39_5$	29	263	328	319
5	647	334	325	30	013	284	279
6	620	266	260	31	5,762	248	243
7	596	204	205	32	512	218	211
8	572	149	150	33	261	192	183
9	545	097	097	34	010	170	158
10	521	052	060	35	4,761	147	138
11	396	1,854	1,872	36	510	123	117
12	270	680	707	37	259	108	100
13	145	563	565	38	009	080	085
14	020	442	457	39	3,758	083?	067
15	8,894	343	346	40	508	067	059
16	770	240	252	41	257	060	049
17	644	152	161	42	007	050	041
18	519	076	082	43	2,756	040	
19	392	1,001	1,010	44	505	032	
20	268	0,937	0,943	45	255	025	
21	143	872	879	46	004	020	
22	017	813	819	47	1,754	016	
23	7,767	717	717	48	503	009	
24	516	628	628	49	253	007	
25	266	550	548				

Tabelle 5. Versuch 4. Anilin–Äthylalkohol, $r = 8{,}59$.

Nr.	x	y	Nr.	x	y
1	—	—	21	$6{,}97_6$	0,855
2	$8{,}58_2$	$2{,}60_6$?	22	845	800
3	539	505?	23	713	750
4	529	413	24	582	686
5	504	313	25	451	643
6	478	262	26	319	597
7	450	197	27	054	519
8	424	145	28	5,792	487
9	360	020	29	529	392
10	292	1,912	30	265	336
11	215	821	31	4,740	252
12	162	730	32	212	183
13	030	584	33	3,685	121
14	7,898	457	34	159	089
15	766	343	35	2,631	068
16	635	240	36	106	043
17	502	150	37	1,580	034
18	372	067	38	053	018
19	240	0,992	39	0,526	009
20	109	918			

Tabelle 6. Versuch 5. Anilin–Äthylalkohol, $r = 6{,}29$.

Nr.	x	y	Nr.	x	y
1	—	—	14	5,208	0,954
2	$6{,}27_5$	2,385	15	4,961	807
3	250	274	16	713	692
4	226	173	17	465	600
5	200	113	18	3,968	432
6	139	1,962	19	472	—
7	077	853	20	2,977	216
8	015	720	21	480	139
9	5,953	612	22	1,983	087
10	829	473	23	488	053
11	705	329	24	0,992	023
12	581	217	25	496	008
13	456	114			

Tabelle 7. Versuch 6. Anilin–Äthylalkohol, $r = 3{,}84_5$.

Nr.	x	y	Nr.	x	y
1	—	—	23	3,293	1,103
2	$3{,}81_9$	2,040	24	267	075
3	795	1,936	25	242	056
4	770	860	26	217	034
5	744	791	27	192	008
6	719	728	28	166	0,981
7	694	669	29	142	961
8	669	622	30	078	908
9	644	571	31	016	857
10	619	529	32	2,953	807
11	593	487	33	890	770
12	569	444	34	765	684
13	543	406	35	638	606
14	518	372	36	513	535
15	493	341	37	261	412
16	468	309	38	010	312
17	443	276	39	1,759	233
18	418	247	40	508	167
19	392	217	41	257	111
20	367	186	42	006	071
21	343	157	43	754	039
22	317	129	44	502	018

Für die Berechnungen zerlegte man das Diagramm in eine hinlängliche Anzahl Vertikalstreifen ΔF von gleicher Breite Δx und fand dann als Grösse des (halben) Meniskusverticalschnittes

$$F = \Sigma \Delta F = \Sigma y \cdot \Delta x \text{ mm}^2 \qquad (1)$$

Tabelle 8.

Versuch	r	H	F	v	m	x'	q/r
1	12,52	2,41	3,40	72,9	0,470	73,1	0,856
2	10,01	2,38	3,27	54,7	0,551	55,1	0,836
3	9,72	2,71	3,98	63,2	0,662	62,6	0,815
4	8,59	2,51	3,68	51,4 (?)	0,620	45,8	0,810
5	6,29	2,43	2,82	28,9	0,693	27,5	0,813?
6	3,84	2,27	1,85	11,1	0,685	10,2	0,792

sowie des Meniskusvolumens[1]

$$v = 2 \sum \Delta F \cdot \left(x + \frac{\Delta x}{2} \right) = 2 q F \text{ mm}^3. \tag{2}$$

Daraus ergab sich auch der Abstand q des Flächenschwerpunktes von der Rohraxe, d.h. der Rotationsradius von F, und dessen relativer Wert q/r. Diese Grösse hat, wie sich leicht zeigen lässt, für Kreise wie für Ellipsen den konstanten Wert

$$\frac{q}{r} = \frac{2}{3} \frac{1}{4 - \pi} = 0{,}7766 \ldots, \tag{3}$$

bei den Meniskuskurven dagegen muss sie von diesem unteren Grenzwerte bei $r = 0$ an gegen den oberen, $q/r = 1$ bei $r = \infty$, ansteigen.

Die zu den Berechnungen erforderliche Randlinienhöhe H kann prinzipiell durch Extrapolation der Kurven gefunden werden. Da aber, wie schon oben bemerkt, diese Extrapolation wegen offenbarer optischer Störungen gerade in den Randpartien zu hohe Werte für H ergeben kann — was besonders in Versuch 4 der Fall ist — so wurde H aus den Messungen von VERSCHAFFELT und VAN DER NOOT (2) durch Interpolation abgeleitet[2].

In Tabelle 8 sind alle Daten für die 6 Versuche zusammengestellt: F, v, H, q/r, die mittlere Meniskushöhe m — diese berechnet wie oben (vgl. 1) angegeben — und das daraus folgende Meniskusvolumen $v' = m\, r^2$, welches gleich v sein soll.

Die unabhängig voneinander ermittelten Volumengrössen v und v' sind in der Tat fast immer sehr nahe gleichgross; eine besonders grobe Differenz findet sich bei Versuch 4, wo die Ordinaten nahe dem Rande sicher wesentlich zu gross ausgefallen sind, so dass sich F und v ebenfalls zu gross ergeben haben.

Der relative Rotationsradius q/r hat den oben vorausgesagten Verlauf.

Der Verlauf von Meniskuskurven

Da ein theoretisch begründeter Ansatz bisher noch nicht aufgestellt worden ist, sollten die vorstehend mitgeteilten Messungen die Grundlage für den Versuch zur Auffindung einer empirischen Näherungsformel liefern, der alle gefundenen Resultate

[1] Der im Volumen auftretende konstante Faktor π ist hier und weiterhin weggelassen worden, weil er weder für q noch sonst in Betracht kommt.

[2] Es ist versucht worden, die Messungen der holländischen Autoren mittels Kathetometers nachzuprüfen; doch konnten genauere Resultate bisher nicht erzielt werden.

sich unterordnen lassen, so dass man aus ihr auch auf den Verlauf solcher Kurven schliessen könnte, welche experimenteller Untersuchung nach dem hier befolgten Verfahren — oder einem anderen — nicht zugänglich sind.

Es erschien zweckmässig, von dem schon vor 100 Jahren durch HAGEN und DESAINS aufgestellten Satze auszugehen, dass die Meniskuskurven sich um so mehr der elliptischen Form nähern, je kleiner der Rohrradius ist. Fasst man diesen als erste Näherung für kleine Radien auf, so sollten die Kurven bei mittelgrossen Radien als "deformierte" Ellipsen anzusehen und ihre Abweichungen von der elliptischen Form durch von Kapillaritätskonstante und Rohrradius abhängige Korrektionsgrössen ausdrückbar sein.

Einer Meniskuskurve mit den Axen H und r korrespondiert eine Ellipse mit dem Ordinatenverlaufe

$$y_e = H\left(1 - \sqrt{1 - \left(\frac{x}{r}\right)^2}\right) = H(1 - w). \tag{4}$$

Setzt man

$$y = y_e \cdot e^{\xi}, \tag{5}$$

so führt bei unseren Versuchen bereits ein relativ einfacher Ansatz

$$\xi = A \cdot \frac{x^p - r^p}{r^p} \tag{6}$$

zu guter Übereinstimmung mit den Experimenten.

Nach eingehender rechnerischer Analyse wurde als universell konstanter Exponent $p = \frac{3}{2}$ gewählt. Der jeder Kurve individuell zugeordnete Zahlenfaktor A, in welchem Kapillaritätskonstante und Radius sich geltend machen müssen, ergab sich als Mittelwert nach Gl. (6) mit Hilfe aller aus Gl. (5) abgeleiteten Exponenten ξ. Zwar zeigt sich deutlich, dass A nur näherungsweise als Konstante betrachtet werden darf, namentlich nicht an den beiden Enden der Kurve, doch weichen die nach Gl. (5) rückwärts berechneten Ordinatenwerte $y_{\text{ber.}}$ von den gefundenen nur sehr wenig ab, wie beispielsweise aus Tabelle 4 hervorgeht, in welcher $y_{\text{ber.}}$ mit angeführt ist. Der Ansatz (6) darf demnach wohl als eine wenigstens vorläufig brauchbare Näherungsformel gelten.

Nun lassen sich diese Factoren A ziemlich gut durch die einfache Beziehung

$$A = \frac{r^2}{10 \cdot a^2} \tag{7}$$

ausdrücken (vgl. Tab. 9).

Darf diese freilich noch etwas grobe Näherung als erlaubt gelten, so kann man nach Gl. (4), (5), (6) und (7) jede noch unbekannte Kurve berechnen. Das aus einer solchen abgeleitete Meniskusvolumen v soll dann gleich mr^2 sein.

Diese Konsequenz ist an zwei hier folgenden Beispielen geprüft worden.

1. Wasser bei 25°, $r = 10$, $a^2 = 14{,}70$, $H = 3{,}73$, $A = 0{,}690$. Aus der Kurve ergab sich $F = 6{,}59$, $v = 105{,}7$, während sich mr^2 zu $1{,}04 \cdot 100 = 104$ berechnet.

2. *o*-Xylol bei 25°, $r = 8{,}0$, $a^2 = 6{,}90$, $H = 2{,}54$, $A = 0.925$. Aus der Kurve folgte $F = 3{,}38$, $v = 43{,}7$, während mr^2 gleich $0{,}675 \cdot 64 = 43{,}2$ ist.

Tabelle 9.

Versuch	A	$\frac{r^2}{10 \cdot a^2}$
1	2,73	2,68
2	1,68	1,70
3	1,42	1,26
4	1,08	1,16
5	0,63	0,63

Zusammenfassung

1. Es wurden sechs Meniskuskurven benetzender Flüssigkeiten photographisch aufgenommen und ausgemessen. Aus dem Verlaufe konnten Meridionalfläche, Volumen und Rotationsradius jedes Meniskus abgeleitet werden. Das gefundene Volumen ergab sich übereinstimmend mit dem unabhängig davon aus Rohrradius und mittlerer Meniskushöhe berechneten Werte.

2. Auf Grund der Versuchsergebnisse wurden empirische Formeln aufgestellt, welche nicht nur den Kurvenverlauf in den hier untersuchten Fällen gut wiedergeben, sondern auch zur Berechnung experimentell nicht bestimmter Meniskuskurven verwendbar zu sein scheinen.

Physikalisch-Chemisches Institut, Universität, Uppsala, Januar 1956.

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